# PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

## PCT

### NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

| | |
|---|---|
| **Date of mailing** (day/month/year)<br>13 November 2000 (13.11.00) | |
| **International application No.**<br>PCT/DE00/00525 | **Applicant's or agent's file reference**<br>0022 |
| **International filing date** (day/month/year)<br>19 February 2000 (19.02.00) | **Priority date** (day/month/year)<br>19 February 1999 (19.02.99) |
| **Applicant**<br>SCHUBERT, Ulrich et al | |

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

18 September 2000 (18.09.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

| | |
|---|---|
| **The International Bureau of WIPO**<br>**34, chemin des Colombettes**<br>**1211 Geneva 20, Switzerland** | Authorized officer<br>Henrik Nyberg |
| Facsimile No.: (41-22) 740.14.35 | Telephone No.: (41-22) 338.83.38 |

# VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS

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09/913927

REC'D 14 JUN 2001
WIPO PCT

# PCT

## INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

(Artikel 36 und Regel 70 PCT)

| Aktenzeichen des Anmelders oder Anwalts | **WEITERES VORGEHEN** | siehe Mitteilung über die Übersendung des internationalen vorläufigen Prüfungsberichts (Formblatt PCT/IPEA/416) |
|---|---|---|
| 0022 | | |
| Internationales Aktenzeichen | Internationales Anmeldedatum *(Tag/Monat/Jahr)* | Prioritätsdatum *(Tag/Monat/Tag)* |
| PCT/DE00/00525 | 19/02/2000 | 19/02/1999 |
| Internationale Patentklassifikation (IPK) oder nationale Klassifikation und IPK | | |
| C07K14/00 | | |
| Anmelder | | |
| SCHUBERT, Ulrich et al. | | |

1. Dieser internationale vorläufige Prüfungsbericht wurde von der mit der internationalen vorläufigen Prüfung beauftragten Behörde erstellt und wird dem Anmelder gemäß Artikel 36 übermittelt.

2. Dieser BERICHT umfaßt insgesamt 7 Blätter einschließlich dieses Deckblatts.

   ☐ Außerdem liegen dem Bericht ANLAGEN bei; dabei handelt es sich um Blätter mit Beschreibungen, Ansprüchen und/oder Zeichnungen, die geändert wurden und diesem Bericht zugrunde liegen, und/oder Blätter mit vor dieser Behörde vorgenommenen Berichtigungen (siehe Regel 70.16 und Abschnitt 607 der Verwaltungsrichtlinien zum PCT).

   Diese Anlagen umfassen insgesamt Blätter.

3. Dieser Bericht enthält Angaben zu folgenden Punkten:

   - I ☒ Grundlage des Berichts
   - II ☐ Priorität
   - III ☐ Keine Erstellung eines Gutachtens über Neuheit, erfinderische Tätigkeit und gewerbliche Anwendbarkeit
   - IV ☒ Mangelnde Einheitlichkeit der Erfindung
   - V ☒ Begründete Feststellung nach Artikel 35(2) hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung
   - VI ☐ Bestimmte angeführte Unterlagen
   - VII ☐ Bestimmte Mängel der internationalen Anmeldung
   - VIII ☐ Bestimmte Bemerkungen zur internationalen Anmeldung

| Datum der Einreichung des Antrags | Datum der Fertigstellung dieses Berichts |
|---|---|
| 18/09/2000 | 12.06.2001 |
| Name und Postanschrift der mit der internationalen vorläufigen Prüfung beauftragten Behörde: | Bevollmächtigter Bediensteter |
| Europäisches Patentamt<br>D-80298 München<br>Tel. +49 89 2399 - 0 Tx: 523656 epmu d<br>Fax: +49 89 2399 - 4465 | Vollbach, S<br>Tel. Nr. +49 89 2399 8715 |

Formblatt PCT/IPEA/409 (Deckblatt) (Januar 1994)

# INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

Internationales Aktenzeichen PCT/DE00/00525

## I. Grundlag d s Berichts

1. Hinsichtlich der **Bestandteile** der internationalen Anmeldung (*Ersatzblätter, die dem Anmeldeamt auf eine Aufforderung nach Artikel 14 hin vorgelegt wurden, gelten im Rahmen dieses Berichts als "ursprünglich eingereicht" und sind ihm nicht beigefügt, weil sie keine Änderungen enthalten (Regeln 70.16 und 70.17)*):
**Beschreibung, Seiten:**

1-17 ursprüngliche Fassung

**Patentansprüche, Nr.:**

1-28 ursprüngliche Fassung

**Zeichnungen, Blätter:**

1/10-10/10 ursprüngliche Fassung

2. Hinsichtlich der **Sprache**: Alle vorstehend genannten Bestandteile standen der Behörde in der Sprache, in der die internationale Anmeldung eingereicht worden ist, zur Verfügung oder wurden in dieser eingereicht, sofern unter diesem Punkt nichts anderes angegeben ist.

Die Bestandteile standen der Behörde in der Sprache: zur Verfügung bzw. wurden in dieser Sprache eingereicht; dabei handelt es sich um

- ☐ die Sprache der Übersetzung, die für die Zwecke der internationalen Recherche eingereicht worden ist (nach Regel 23.1(b)).
- ☐ die Veröffentlichungssprache der internationalen Anmeldung (nach Regel 48.3(b)).
- ☐ die Sprache der Übersetzung, die für die Zwecke der internationalen vorläufigen Prüfung eingereicht worden ist (nach Regel 55.2 und/oder 55.3).

3. Hinsichtlich der in der internationalen Anmeldung offenbarten **Nucleotid- und/oder Aminosäuresequenz** ist die internationale vorläufige Prüfung auf der Grundlage des Sequenzprotokolls durchgeführt worden, das:

- ☐ in der internationalen Anmeldung in schriftlicher Form enthalten ist.
- ☐ zusammen mit der internationalen Anmeldung in computerlesbarer Form eingereicht worden ist.
- ☐ bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.
- ☐ bei der Behörde nachträglich in computerlesbarer Form eingereicht worden ist.
- ☐ Die Erklärung, daß das nachträglich eingereichte schriftliche Sequenzprotokoll nicht über den Offenbarungsgehalt der internationalen Anmeldung im Anmeldezeitpunkt hinausgeht, wurde vorgelegt.
- ☐ Die Erklärung, daß die in computerlesbarer Form erfassten Informationen dem schriftlichen Sequenzprotokoll entsprechen, wurde vorgelegt.

4. Aufgrund der Änderungen sind folgende Unterlagen fortgefallen:

- ☐ Beschreibung, Seiten:
- ☐ Ansprüche, Nr.:
- ☐ Zeichnungen, Blatt:

5. ☐ Dieser Bericht ist ohne Berücksichtigung (von einigen) der Änderungen erstellt worden, da diese aus den angegebenen Gründen nach Auffassung der Behörde über den Offenbarungsgehalt in der ursprünglich eingereichten Fassung hinausgehen (Regel 70.2(c)).

   *(Auf Ersatzblätter, die solche Änderungen enthalten, ist unter Punkt 1 hinzuweisen;sie sind diesem Bericht beizufügen).*

6. Etwaige zusätzliche Bemerkungen:

**IV. Mangelnde Einheitlichkeit der Erfindung**

1. Auf die Aufforderung zur Einschränkung der Ansprüche oder zur Zahlung zusätzlicher Gebühren hat der Anmelder:

   ☐ die Ansprüche eingeschränkt.

   ☐ zusätzliche Gebühren entrichtet.

   ☐ zusätzliche Gebühren unter Widerspruch entrichtet.

   ☐ weder die Ansprüche eingeschränkt noch zusätzliche Gebühren entrichtet.

2. ☐ Die Behörde hat festgestellt, daß das Erfordernis der Einheitlichkeit der Erfindung nicht erfüllt ist, und hat gemäß Regel 68.1 beschlossen, den Anmelder nicht zur Einschränkung der Ansprüche oder zur Zahlung zusätzlicher Gebühren aufzufordern.

3. Die Behörde ist der Auffassung, daß das Erfordernis der Einheitlichkeit der Erfindung nach den Regeln 13.1, 13.2 und 13.3

   ☐ erfüllt ist

   ☐ aus folgenden Gründen nicht erfüllt ist:

4. Daher wurde zur Erstellung dieses Berichts eine internationale vorläufige Prüfung für folgende Teile der internationalen Anmeldung durchgeführt:

   ☒ alle Teile.

   ☐ die Teile, die sich auf die Ansprüche Nr. beziehen.

**V. Begründete Feststellung nach Artikel 35(2) hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung**

# INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

Internationales Aktenzeichen PCT/DE00/00525

1. Feststellung

| | | | |
|---|---|---|---|
| Neuheit (N) | Ja: | Ansprüche | 2-28 |
| | Nein: | Ansprüche | 1 |
| Erfinderische Tätigkeit (ET) | Ja: | Ansprüche | |
| | Nein: | Ansprüche | 2-28 |
| Gewerbliche Anwendbarkeit (GA) | Ja: | Ansprüche | 1-28 |
| | Nein: | Ansprüche | |

2. Unterlagen und Erklärungen
**siehe Beiblatt**

**Zu Punkt IV**

Mangelnde Einheitlichkeit der Erfindung

Da der Anspruch 1 der vorliegenden Anmeldung nicht die Erfordernisse des Artikel 33(2) PCT erfüllt (siehe Punkt V) ergibt sich, daß die Peptide der Ansprüche 2 und 3 nicht mehr durch ein einheitliches Konzept miteinander verbunden sind. Der ganze Anspruchsatz zerfällt in eine Vielfalt von Erfindungsgruppen entsprechend der Anzahl der beanspruchten Peptide. Weiterhin folgt, daß ein einheitliches Konzept zwischen der Verwendung und Herstellung auf der einen Seite und den verschiedenen Verwendungen auf der anderen Seite nicht existiert.

Gegenwärtig wird dieser unter Regel 13.1-13.3 PCT erhobene Einwand nicht verfolgt, da weitere ernsthafte Einwände für den gesamten Anspruchsatz gelten.

**Zu Punkt V**

Begründete Feststellung nach Regel 66.2(a)(ii) hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung

Die vorliegende Anmeldung betrifft synthetische Peptide des regulatorischen Virusproteins R (Vpr) des HIV. Beansprucht werden ferner Verfahren zur Herstellung und Verwendung der Peptide.

Folgende Dokumente werden im vorliegenden Bescheid zitiert:

D1: WO 98 44945 A (IMMUNE RESPONSE CORP INC) 15. Oktober 1998 (1998-10-15)

D2: DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 5. Februar 1999 (1999-02-05) SCHULER W ET AL: 'NMR structure of the (52-96) C-terminal domain of the HIV-1 regulatory protein Vpr: Molecular insights into its biological functions.' Database accession no. PREV199900140755 XP002145860 & JOURNAL OF MOLECULAR BIOLOGY, Bd. 285, Nr. 5, 5. Februar 1999 (1999-02-05), Seiten 2105-2117, ISSN: 0022-2836

D3: Z. LUO ET AL.: 'Structural Studies of Synthetic Peptide Fragments Derived from the HIV-1 Vpr Protein' BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Bd. 244, Nr. 3, 27. März 1998 (1998-03- 27), Seiten 732-

736, XP002145859 ORLANDO, FL US

Alle Dokumente beschreiben Vpr Peptide des HIV 1. Obwohl das Herstellungverfahren irrelevant ist für die Beurteilung der Neuheit, sind in den zitierten Dokumenten Peptide beschrieben, die chemisch synthetisiert sind. Daher ist der Anspruch 1 nicht mehr neu. Für die Peptide der Ansprüche 2 und 3, sofern sie neu sind, kann erfinderische Tätigkeit nicht anerkannt werden. Die Bereitstellung von Peptiden eines bekannten Proteins, welche zudem nur geringfügig von denen des Standes der Technik abweichen, kann ohne jede erfinderische Tätigkeit vom Fachmann geleistet werden. Da die Produktansprüche auch keineswegs auf ein 96 Aminosäure langes Peptid beschränkt sind, kann eine potentielle überraschende Wasserlöslichkeit den Ansprüchen keine erfinderische Tätigkeit verleihen. Daher sind die Produkte unter Artikel 33(3) PCT nicht gewährbar.
In der Beschreibung der vorliegenden Anmeldung gibt die Anmelderin als subjektive Problem die Bereitstellung "eines Syntheseweg für Vrp-Peptide im mg Maßstab" an. Die Lösung ist die Synthese der C-terminalen Vpr-Peptide an einem Serin-Harz mit Hilfe eines Perkin-Elmar-Synthesizer. Es wird angegeben, daß im Gegensatz zu den bisherigen Peptiden, die der vorliegenden Anmeldung wasserlöslich sind und auch in hoher Konzentration keiner Proteinaggregation unterliegen. Die Proteine der Dokumente D1 bis D3 erfüllen auch diese Aufgabe. Daher gibt es keinerlei Basis für Produktansprüche.
Sollte die Erfindung allerdings in einer spezifischen Methode zur Herstellung liegen- was derzeit nicht gesehen wird- dann ist die Anmeldung auf eben dieses Verfahren zu beschränken. Allgemeine Herstellungsverfahren gemäß der Ansprüche 4-6 sind im Hinblick auf die allgemeinen Kenntnisse der chemischen Synthese unter Artikel 33 (3) nicht gewährbar.

Betreffend der Verfahrensansprüche 7-28 wird die Auffassung vertreten, daß diese nicht über das was der Fachmann machen würde hinausgehen. Für die meisten der beanspruchten Verwendungen gibt es ohnehin keine Stützung in der Beschreibung.

Zusammenfassend läßt sich sagen, daß die allgemeinen Peptide nicht neu sind, die spezifischen Peptiden die Erfordernisse des Artikel 33(3) PCT nicht erfüllen und die Verwendungen üblich sind für den Fachmann.

Daher kann kein Gegenstand erkannt werden, der eine Basis für einen erlaubbaren Anspruchsatz bilden könnte.

VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS

# PCT

## INTERNATIONALER RECHERCHENBERICHT

(Artikel 18 sowie Regeln 43 und 44 PCT)

| Aktenzeichen des Anmelders oder Anwalts<br>0022 | **WEITERES VORGEHEN** siehe Mitteilung über die Übermittlung des internationalen Recherchenberichts (Formblatt PCT/ISA/220) sowie, soweit zutreffend, nachstehender Punkt 5 | |
|---|---|---|
| Internationales Aktenzeichen<br>PCT/DE 00/ 00525 | Internationales Anmeldedatum *(Tag/Monat/Jahr)*<br>19/02/2000 | (Frühestes) Prioritätsdatum *(Tag/Monat/Jahr)*<br>19/02/1999 |
| Anmelder<br>SCHUBERT, Ulrich et al. | | |

Dieser internationale Recherchenbericht wurde von der Internationalen Recherchenbehörde erstellt und wird dem Anmelder gemäß Artikel 18 übermittelt. Eine Kopie wird dem Internationalen Büro übermittelt.

Dieser internationale Recherchenbericht umfaßt insgesamt 4 Blätter.

[X] Darüber hinaus liegt ihm jeweils eine Kopie der in diesem Bericht genannten Unterlagen zum Stand der Technik bei.

1. **Grundlage des Berichts**

   a. Hinsichtlich der **Sprache** ist die internationale Recherche auf der Grundlage der internationalen Anmeldung in der Sprache durchgeführt worden, in der sie eingereicht wurde, sofern unter diesem Punkt nichts anderes angegeben ist.

   [ ] Die internationale Recherche ist auf der Grundlage einer bei der Behörde eingereichten Übersetzung der internationalen Anmeldung (Regel 23.1 b)) durchgeführt worden.

   b. Hinsichtlich der in der internationalen Anmeldung offenbarten **Nucleotid- und/oder Aminosäuresequenz** ist die internationale Recherche auf der Grundlage des Sequenzprotokolls durchgeführt worden, das

   [ ] in der internationalen Anmeldung in Schriftlicher Form enthalten ist.

   [ ] zusammen mit der internationalen Anmeldung in computerlesbarer Form eingereicht worden ist.

   [X] bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.

   [X] bei der Behörde nachträglich in computerlesbarer Form eingereicht worden ist.

   [X] Die Erklärung, daß das nachträglich eingereichte schriftliche Sequenzprotokoll nicht über den Offenbarungsgehalt der internationalen Anmeldung im Anmeldezeitpunkt hinausgeht, wurde vorgelegt.

   [X] Die Erklärung, daß die in computerlesbarer Form erfaßten Informationen dem schriftlichen Sequenzprotokoll entsprechen, wurde vorgelegt.

2. [ ] **Bestimmte Ansprüche haben sich als nicht recherchierbar erwiesen** (siehe Feld I).

3. [ ] **Mangelnde Einheitlichkeit der Erfindung** (siehe Feld II).

4. Hinsichtlich der **Bezeichnung der Erfindung**

   [X] wird der vom Anmelder eingereichte Wortlaut genehmigt.

   [ ] wurde der Wortlaut von der Behörde wie folgt festgesetzt:

5. Hinsichtlich der **Zusammenfassung**

   [X] wird der vom Anmelder eingereichte Wortlaut genehmigt.

   [ ] wurde der Wortlaut nach Regel 38.2b) in der in Feld III angegebenen Fassung von der Behörde festgesetzt. Der Anmelder kann der Behörde innerhalb eines Monats nach dem Datum der Absendung dieses internationalen Recherchenberichts eine Stellungnahme vorlegen.

6. Folgende Abbildung der **Zeichnungen** ist mit der Zusammenfassung zu veröffentlichen: Abb. Nr. _____

   [ ] wie vom Anmelder vorgeschlagen [X] keine der Abb.

   [ ] weil der Anmelder selbst keine Abbildung vorgeschlagen hat.

   [ ] weil diese Abbildung die Erfindung besser kennzeichnet.

# INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/DE 00/00525

**A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES**
IPK 7 C07K14/16 G01N33/68 C07K16/10 A61P31/18

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

**B. RECHERCHIERTE GEBIETE**

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole )
IPK 7 C07K G01N

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)
BIOSIS, EPO-Internal, WPI Data

**C. ALS WESENTLICH ANGESEHENE UNTERLAGEN**

| Kategorie° | Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile | Betr. Anspruch Nr. |
|---|---|---|
| X | WO 98 44945 A (IMMUNE RESPONSE CORP INC) 15. Oktober 1998 (1998-10-15)<br>Seite 9, Zeile 1 - Zeile 4; Ansprüche 24-27<br>Seite 15, Zeile 29 -Seite 16, Zeile 9<br>--- | 1,2,7,8, 10-12, 25,26 |
| X | WO 95 26361 A (BIOMOLECULAR RES INST LTD ;AZAD AHMED A (AU); MACREADIE IAN G (AU)) 5. Oktober 1995 (1995-10-05)<br>Seite 3, Zeile 1 - Zeile 26<br>Seite 4, Zeile 6 -Seite 5, Zeile 10<br>Seite 6, Zeile 16 - Zeile 20; Ansprüche; Beispiel 6<br>---<br>-/-- | 1,2,4,7, 8,12,16, 19 |

[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[X] Siehe Anhang Patentfamilie

° Besondere Kategorien von angegebenen Veröffentlichungen :
"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist
"E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist
"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)
"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht
"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist
"T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist
"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden
"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist
"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

| Datum des Abschlusses der internationalen Recherche | Absendedatum des internationalen Recherchenberichts |
|---|---|
| 28. August 2000 | 15/09/2000 |

| Name und Postanschrift der Internationalen Recherchenbehörde | Bevollmächtigter Bediensteter |
|---|---|
| Europäisches Patentamt, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31–70) 340–2040, Tx. 31 651 epo nl,<br>Fax: (+31–70) 340–3016 | Fuhr, C |

1

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

| Kategorie° | Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile | Betr. Anspruch Nr. |
|---|---|---|
| X | DATABASE BIOSIS 'Online!<br>BIOSCIENCES INFORMATION SERVICE,<br>PHILADELPHIA, PA, US;<br>5. Februar 1999 (1999-02-05)<br>SCHULER W ET AL: "NMR structure of the (52-96) C-terminal domain of the HIV-1 regulatory protein Vpr: Molecular insights into its biological functions."<br>Database accession no. PREV199900140755<br>XP002145860<br>Zusammenfassung<br>& JOURNAL OF MOLECULAR BIOLOGY,<br>Bd. 285, Nr. 5,<br>5. Februar 1999 (1999-02-05), Seiten 2105-2117,<br>ISSN: 0022-2836<br>--- | 1,2 |
| P,X | DATABASE BIOSIS 'Online!<br>BIOSCIENCES INFORMATION SERVICE,<br>PHILADELPHIA, PA, US;<br>November 1999 (1999-11)<br>CORNILLE F ET AL: "Efficient solid-phase synthesis of Vpr from HIV-1 using low quantities of uniformly 13C-, 15N-labeled amino acids for NMR structural studies."<br>Database accession no. PREV200000001193<br>XP002145861<br>Zusammenfassung<br>& JOURNAL OF PEPTIDE RESEARCH,<br>Bd. 54, Nr. 5, November 1999 (1999-11),<br>Seiten 427-435,<br>ISSN: 1397-002X<br>--- | 1,2 |
| P,X | DATABASE BIOSIS 'Online!<br>BIOSCIENCES INFORMATION SERVICE,<br>PHILADELPHIA, PA, US;<br>Dezember 1999 (1999-12)<br>WECKER K ET AL: "NMR structure of the (1-51) N-terminal domain of the HIV-1 regulatory protein Vpr."<br>Database accession no. PREV200000048307<br>XP002145862<br>Zusammenfassung<br>& EUROPEAN JOURNAL OF BIOCHEMISTRY,<br>Bd. 266, Nr. 2, Dezember 1999 (1999-12),<br>Seiten 359-369,<br>ISSN: 0014-2956<br>---<br>-/-- | 1,2 |

1

Internationales Aktenzeichen
PCT/DE 00/00525

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

| Kategorie° | Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile | Betr. Anspruch Nr. |
|---|---|---|
| X | Z. LUO ET AL.: "Structural Studies of Synthetic Peptide Fragments Derived from the HIV-1 Vpr Protein" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Bd. 244, Nr. 3, 27. März 1998 (1998-03-27), Seiten 732-736, XP002145859 ORLANDO, FL US Seite 734, rechte Spalte, Absatz 1; Abbildung 1 ----- | 1,2,7,8 |

# INTERNATIONAL SEARCH REPORT

rmation on patent family members

'nternational Application No
PCT/DE 00/00525

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9844945 A | 15-10-1998 | AU 7101798 A | 30-10-1998 |
| WO 9526361 A | 05-10-1995 | AU 697620 B | 15-10-1998 |
| | | AU 2063495 A | 17-10-1995 |
| | | CA 2186398 A | 05-10-1995 |
| | | EP 0753006 A | 15-01-1997 |
| | | JP 9511395 T | 18-11-1997 |

09/913927
Translation
05-00

# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| Applicant's or agent's file reference 0022 | FOR FURTHER ACTION | See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) |
|---|---|---|
| International application No. PCT/DE00/00525 | International filing date (*day/month/year*) 19 February 2000 (19.02.00) | Priority date (*day/month/year*) 19 February 1999 (19.02.99) |
| International Patent Classification (IPC) or national classification and IPC C07K 14/00 | | |
| Applicant SCHUBERT, Ulrich | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

| Date of submission of the demand | Date of completion of this report |
|---|---|
| 18 September 2000 (18.09.00) | 12 June 2001 (12.06.2001) |
| Name and mailing address of the IPEA/EP<br><br>Facsimile No. | Authorized officer<br><br>Telephone No. |

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/DE00/00525

## I. Basis of the report

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed

☒ the description:

pages 1-17, as originally filed

pages _____, filed with the demand

pages _____, filed with the letter of _____

☒ the claims:

pages 1-28, as originally filed

pages _____, as amended (together with any statement under Article 19

pages _____, filed with the demand

pages _____, filed with the letter of _____

☒ the drawings:

pages 1/10-10/10, as originally filed

pages _____, filed with the demand

pages _____, filed with the letter of _____

☐ the sequence listing part of the description:

pages _____, as originally filed

pages _____, filed with the demand

pages _____, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages _____

☐ the claims, Nos. _____

☐ the drawings, sheets/fig _____

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rule 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

**IV. Lack of unity of invention**

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☐ not complied with for the following reasons:

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. _____.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Claims | 2-28 | YES |
| | Claims | 1 | NO |
| Inventive step (IS) | Claims | | YES |
| | Claims | 2-28 | NO |
| Industrial applicability (IA) | Claims | 1-28 | YES |
| | Claims | | NO |

2. Citations and explanations

The present application pertains to synthetic peptides of the HIV regulatory virus protein R (Vpr). Processes for preparing the peptides and uses thereof are also claimed.

The following documents are referred to in the present report:

D1: WO-A-98/44945 (IMMUNE RESPONSE CORP. INC.) 15 October 1998 (1998-10-15)

D2: DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 5 February 1999 (1999-02-05) SCHULER W ET AL.: 'NMR structure of the (52-96) C-terminal domain of the HIV-1 regulatory protein Vpr: Molecular insights into its biological functions.' Database accession no. PREV199900140755 XP002145860 & JOURNAL OF MOLECULAR BIOLOGY, vol. 285, no. 5, 5 February 1999 (1999-02-05), pages 2105-2117, ISSN: 0022-2836

D3: Z. LUO ET AL.: 'Structural Studies of Synthetic Peptide Fragments Derived from the HIV-1 Vpr Protein' BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 244, no. 3, 27 March 1998 (1998-03-27), pages 732-736, XP002145859 ORLANDO, FL, US

All the documents describe HIV-1 Vpr peptides. Although

**INTERNATIONAL PRELIMINARY EXAMINATION REPORT**

International application No.
PCT/DE 00/00525

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Claims | 2-28 | **YES** |
| | Claims | 1 | **NO** |
| Inventive step (IS) | Claims | | **YES** |
| | Claims | 2-28 | **NO** |
| Industrial applicability (IA) | Claims | 1-28 | **YES** |
| | Claims | | **NO** |

2. Citations and explanations

The present application pertains to synthetic peptides of the HIV regulatory virus protein R (Vpr). A process for preparing the peptides and the use thereof are also claimed.

The following documents are referred to in the present report:

D1: WO-A-98/44945 (IMMUNE RESPONSE CORP. INC.) 15 October 1998 (1998-10-15)

D2: DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 5 February 1999 (1999-02-05) SCHULER W ET AL.: 'NMR structure of the (52-96) C-terminal domain of the HIV-1 regulatory protein Vpr: Molecular insights into its biological functions.' Database accession no. PREV199900140755 XP002145860 & JOURNAL OF MOLECULAR BIOLOGY, vol. 285, no. 5, 5 February 1999 (1999-02-05), pages 2105-2117, ISSN: 0022-2836

D3: Z. LUO ET AL.: 'Structural Studies of Synthetic Peptide Fragments Derived from the HIV-1 Vpr Protein' BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 244, no. 3, 27 March 1998 (1998-03-27), pages 732-736, XP002145859 ORLANDO, FL, US

All the documents describe HIV-1 Vpr peptides. Although the process of preparation is not relevant to evaluation of novelty, the citations describe peptides that are synthesized chemically. Consequently, Claim 1 is not novel.

To the extent that the peptides described in Claims 2 and 3 are novel, they cannot be acknowledged to involve an inventive step. Peptides of a known protein which, moreover, differ only slightly from prior art peptides, may be prepared by a person skilled in the art without inventive input. Since the product claims are also by no means limited to a peptide 96 amino acids in length, unexpected potential water solubility cannot substantiate inventive step. Therefore, the products are not allowable under PCT Article 33(3).

In the present description the applicant states the subjective problem to consist in providing "a synthetic pathway for Vpr peptides in amounts measured in milligrams". The solution consists in the synthesis of C-terminal Vpr peptides on a serine resin using a Perkin-Elmar synthesizer. The applicant indicates that, unlike existing peptides, those described in the present application are water-soluble and not subject to protein aggregation even at high concentrations. Since the proteins described in D1-D3 also solve this problem, the present product claims lack any basis.

If, however, the invention consists in a specific preparation process (which is not apparent at present), the application should be restricted to this process. In light of universally known chemical synthesis procedures, general methods of preparation as per Claims 4-6 are not allowable under PCT Article 33(3).

With respect to process Claims 7-28, the examiner is of the opinion that these do not go beyond routine professional practice. In any case, the majority of the claimed uses are not supported by the description.

To summarize, the general peptides are not novel, the specific peptides do not meet the requirements of PCT Article 33(3) and the uses are routine for a person skilled in the art.

Therefore, subject matter that could form the basis for an allowable set of claims cannot be discerned.

**Supplemental Box**
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: IV.4

Since Claim 1 of the present application does not meet the requirements of PCT Article 33(2) (see Box V), the peptides described in Claims 2 and 3 are no longer linked by a unified concept. The entire set of claims is thus reduced to a number of groups of inventions corresponding to the number of peptides claimed. Further, it follows that there is no unified concept linking either use and production or the various uses.

This objection, raised under PCT Rule 13.1 to 13.3, has not been pursued, since there are further serious objections to the set of claims as a whole.

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro

(43) Internationales Veröffentlichungsdatum
24. August 2000 (24.08.2000)

PCT

(10) Internationale Veröffentlichungsnummer
WO 00/49038 A3

(51) Internationale Patentklassifikation[7]: C07K 14/16, G01N 33/68, C07K 16/10, A61P 31/18

(21) Internationales Aktenzeichen: PCT/DE00/00525

(22) Internationales Anmeldedatum:
19. Februar 2000 (19.02.2000)

(25) Einreichungssprache: Deutsch

(26) Veröffentlichungssprache: Deutsch

(30) Angaben zur Priorität:
199 08 752.0 19. Februar 1999 (19.02.1999) DE
199 08 766.0 19. Februar 1999 (19.02.1999) DE

(71) Anmelder und
(72) Erfinder: **SCHUBERT, Ulrich** [DE/DE]; Jenaische Strasse 51, D-07407 Uhlstädt (DE). **HENKLEIN, Peter** [DE/DE]; Schulze-Boysen-Strasse 25, D-10365 Berlin (DE). **WRAY, Victor** [GB/DE]; Elbinger Strasse 6, D-38302 Wolfenbüttel (DE).

(74) Anwalt: **WEHLAN, Helmut**; Paul-Gesche-Strasse 1, D-10315 Berlin (DE).

(81) Bestimmungsstaaten *(national)*: JP, US.

(84) Bestimmungsstaaten *(regional)*: europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Veröffentlicht:
— *Mit internationalem Recherchenbericht.*

(88) Veröffentlichungsdatum des internationalen Recherchenberichts: 1. März 2001

*Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.*

(54) Title: SYNTHETIC PEPTIDE OF REGULATORY VIRUS PROTEIN R (VPR) OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) AND THE UTILIZATION THEREOF

(54) Bezeichnung: SYNTHETISCHE PEPTIDE DES REGULATORISCHEN VIRUSPROTEINS R (VPR) DES HUMANEN IMMUNDEFIZIENZVIRUS TYP 1 (HIV-1) UND IHRE VERWENDUNG

(57) Abstract: The invention relates to synthetic (s) peptides of regulatory virus protein R (Vpr) of human immunodeficiency virus type 1(HIV-1), especially the total chemical synthesis of 96 aminoacid long Vpr protein (sVpr1-96), a 47 aminoacid long N terminal fragment (sVpr1-47) and a 49 aminoacid long C terminal fragment thereof (sVpr48-96) and to fragments sVpr1-20 and sVpr21-40, in addition to other fragments with approximately 15 aminoacids. Said products are used as HIV-1 regulatory proteins in biological assays and in the analysis of molecular structure and physico-chemical properties of Vpr and its domains or in the production of antibodies directed against Vpr-peptide sequences.

(57) Zusammenfassung: Die Erfindung betrifft synthetische (s) Peptide des regulatorischen Virusproteins R (Vpr) des Humanen Immundefizienzvirus Typ 1 (HIV-1), insbesondere die chemische Totalsynthese des 96 Aminosäuren langen Vpr-Proteins (sVpr1-96), eines 47 Aminosäuren langen N-terminalen (sVpr1-47), eines 49 Aminosäuren langen C-terminalen Fragmentes davon (sVpr48-96) sowie der Fragmente sVpr1-20 und sVpr21-40 und weiterer Fragmente mit etwa 15 Aminosäuren. Als HIV-1-regulatorische Proteine finden die Produkte Verwendung in biologischen Assays, in der Analyse der molekularen Struktur und der physikochemischen Eigenschaften von Vpr und dessen Domänen oder zur Erzeugung von Antikörpern gegen Vpr-Peptidsequenzen.

WO 00/49038 A3

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

BERICHTIGTE FASSUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro

(43) Internationales Veröffentlichungsdatum
24. August 2000 (24.08.2000)

PCT

(10) Internationale Veröffentlichungsnummer
WO 00/49038 A3

(51) Internationale Patentklassifikation[7]: C07K 14/16, G01N 33/68, C07K 16/10, A61P 31/18

(21) Internationales Aktenzeichen: PCT/DE00/00525

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(71) Anmelder und
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(74) Anwalt: **WEHLAN, Helmut**; Paul-Gesche-Strasse 1, D-10315 Berlin (DE).

(81) Bestimmungsstaaten *(national)*: JP, US.

(84) Bestimmungsstaaten *(regional)*: europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Veröffentlicht:
— *Mit internationalem Recherchenbericht.*

(88) Veröffentlichungsdatum des internationalen Recherchenberichts: 1. März 2001

(48) Datum der Veröffentlichung dieser berichtigten Fassung: 17. Mai 2001

(15) Informationen zur Berichtigung:
siehe PCT Gazette Nr. 20/2001 vom 17. Mai 2001, Section II

*Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.*

(54) Title: SYNTHETIC PEPTIDE OF REGULATORY VIRUS PROTEIN R (VPR) OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) AND THE UTILIZATION THEREOF

(54) Bezeichnung: SYNTHETISCHE PEPTIDE DES REGULATORISCHEN VIRUSPROTEINS R (VPR) DES HUMANEN IMMUNDEFIZIENZVIRUS TYP 1 (HIV-1) UND IHRE VERWENDUNG

(57) Abstract: The invention relates to synthetic (s) peptides of regulatory virus protein R (Vpr) of human immunodeficiency virus type 1(HIV-1), especially the total chemical synthesis of 96 aminoacid long Vpr protein (sVpr1-96), a 47 aminoacid long N terminal fragment (sVpr1-47) and a 49 aminoacid long C terminal fragment thereof (sVpr48-96) and to fragments sVpr1-20 and sVpr21-40, in addition to other fragments with approximately 15 aminoacids. Said products are used as HIV-1 regulatory proteins in biological assays and in the analysis of molecular structure and physico-chemical properties of Vpr and its domains or in the production of antibodies directed against Vpr-peptide sequences.

(57) Zusammenfassung: Die Erfindung betrifft synthetische (s) Peptide des regulatorischen Virusproteins R (Vpr) des Humanen Immundefizienzvirus Typ 1 (HIV-1), insbesondere die chemische Totalsynthese des 96 Aminosäuren langen Vpr-Proteins (sVpr1-96), eines 47 Aminosäuren langen N-terminalen (sVpr1-47), eines 49 Aminosäuren langen C-terminalen Fragmentes davon (sVpr48-96) sowie der Fragmente sVpr1-20 und sVpr21-40 und weiterer Fragmente mit etwa 15 Aminosäuren. Als HIV-1-regulatorische Proteine finden die Produkte Verwendung in biologischen Assays, in der Analyse der molekularen Struktur und der physikochemischen Eigenschaften von Vpr und dessen Domänen oder zur Erzeugung von Antikörpern gegen Vpr-Peptidsequenzen.

WO 00/49038 A3

# Synthetische Peptide des regulatorischen Virusproteins R (Vpr) des Humanen Immundefizienzvirus Typ 1 (HIV-1) und ihre Verwendung

## Beschreibung

Die Erfindung betrifft synthetische (s) Peptide des regulatorischen Virusproteins R (Vpr) des Humanen Immundefizienzvirus Typ 1 (HIV-1), insbesondere die chemische Totalsynthese des 96 Aminosäure langen Vpr-Proteins (sVpr1-96) sowie seiner Sequenzen. Als synthetische Vpr-Peptide finden sie Verwendung in biologischen Assays, in der Analyse der molekularen Struktur und den physikochemischen Eigenschaften von Vpr und dessen Domänen sowie zur Erzeugung von Antikörpern gegen Vpr-Peptidsequenzen.

Die bislang einzige *in vitro* charakterisierte biochemische Aktivität von Vpr ist die eines Kationen-selektiven Ionenkanals (Piller *et al.*, 1996, - Literaturverzeichnis am Ende der Ausführungsbeispiele). Diese Arbeiten basierten auf der Annahme, daß die C-terminale alpha Helix (Positionen 46 bis 71 in Vpr), welche Ähnlichkeiten zu der Bienengift-Komponente Melittin besitzt, als Transmembrananker eine Membranpore ausbilden kann. Tatsächlich konnte rekombinantes, in *Escherichia (E.) coli* exprimiertes Vpr in künstlichen planaren Lipidbilayern rekonstituiert werden. Dadurch wurde eine durch das Membranpotential regulierbare Ionenkanalaktivität ermittelt, deren Regulierbarkeit von der basischen C-terminalen Region abhängt, welche mit der negativ geladenen zytoplasmatischen Seite der Zellmembran in Wechselwirkung treten soll.

Es liegen Hinweise für Homooligomerisierung von Vpr vor: Ein rekombinantes Vpr-Fusionsprotein bildet oligomere Strukturen mit Molekulargewichten von >100 kDa (Zhao *et al.*, 1994b), eine Beobachtung, die bislang an viralen Vpr nicht bestätigt wurde.

Untersuchungen zur molekularen Struktur von Vpr wurden durch zwei Gruppen mittels Sekundärstruktur-Analysen an kurzen Vpr-Peptiden durchgeführt: NMR-Studien an überlappenden Peptiden in wässerigem Trifluorethanol (TFE) sowie in Natriumdodecylsulfat(SDS)-Mizellen identifizierten alpha-helikale Regionen in den Vpr-Positionen 50-82. (Yao *et al.*, 1998). Das Potential zur Helix-Bildung in der C-terminalen als auch der N-terminalen Region von Vpr wurde zuvor von verschiedenen Autoren vorhergesagt (Mahalingam *et al.*, 1995a-d: Yao *et al.*, 1995: Wang *et al.*, 1996b). Neuere Studien mittels CD-Spektroskopie in TFE-haltigen Lösungen an 25 Aminosäure langen Peptiden (Luo *et al.*, 1998) zeigten erste experimentelle Hinweise für die Existenz der N- und C-terminalen Helices in Vpr. Zahlreiche und zum Teil in ihrer Aussage kontroverse Mutationsanalysen haben versucht, die

verschiedenen Primär- und Sekundärstrukturen einzelnen biologischen Aktivitäten von Vpr zuzuordnen (Mahalingam *et al.*, 1995a-d, 1997; Wang *et al.*, 1996a,b; Nie *et al.*, 1998; Di Marzio *et al.*, 1995).

Über die chemische Vollsynthese eines Vpr-Proteins wurde erstmals 1997 von Rocquigny und Mitarbeitern berichtet. Die Autoren beschrieben die Synthese eines 96 Aminosäure großen Peptides, welches von dem Virusisolat HIV-$1_{89.6}$ (Collman *et al.* 1992) abstammt. Neben den in dieser Arbeit beschriebenen Nachteilen (siehe im weiteren Text) ist dieses Protein in 9 Aminosäurepositionen unterschiedlich zu Vpr von HIV-$1_{NL4-3}$, dessen Darstellung in der vorliegenden Erfindungsbeschreibung erstmalig berichtet wird. Somit besteht eine 10%-ige Divergenz zwischen den bereits beschriebenen (Rocquigny *et al.*, 1997) und dem in den vorliegenden Verfahren dargestellten Produkten, welche die Gesamt- und Teilsequenzen des Vpr-Proteins von HIV-$1_{NL4-3}$ (Adachi *et al.*, 1986) betreffen.

Rocquigny und Mitarbeitern (1997) geben keine Angaben über die Reinheit sowie die physikochemischen Eigenschaften des Vpr-Peptides an. Es wird lediglich mittels der Far-Westernblot Technik gezeigt, daß SDS-denaturiertes Vpr-Peptid mit dem viralen Nukleoprotein NCp7 des gleichen HIV-1-Isolates in Wechselwirkung tritt. Dieser Befund der NCp7-Vpr-Wechselwirkung konnte bislang von keiner der zahlreichen anderen auf dem Vpr-Gebiet forschenden Gruppen bestätigt werden. Wesentlicher Nachteil dieser Vpr-Synthese ist die Tatsache, daß für dieses Peptid keine der beschriebenen biologischen Aktivitäten durch die Autoren gezeigt wurde. Insbesondere wird gezeigt, daß dieses Vpr-Peptid nicht an $p6^{Gag}$ bindet, eine weithin akzeptierte Eigenschaft von Vpr (Paxton *et al.*, 1993; Lavallee *et al.*, 1994; Kondo *et al.*, 1995; Lu *et al.*, 1995; Kondo und Göttlinger, 1996). Darüber hinaus wird beschrieben, daß dieses Peptid keine Oligomeren bildet, und es liegen Hinweise vor, daß dieses Peptid in rein wässerigem System unlöslich ist. Von dem gleichen Labor wird in einer weiteren Studie (Roques *et al.*, 1997) ein Modell der Vpr-NCp7-Wechselwirkung vorgestellt, welches auf Strukturanalysen an Teilsequenzen dieser Peptide basiert. Die Daten dazu werden jedoch in dieser Arbeit oder anderen Veröffentlichungen der Autoren nicht näher beschrieben.

Teilsequenzen von Vpr (Positionen 50-75, 50-82 und 59-86) wurden für NMR-Studien an synthetischen Peptiden eingesetzt (Yao *et al.*, 1998). Eine andere Gruppe hat zwei 25 Aminosäure lange Peptide aus den Bereichen der vorhergesagten alpha-helikalen Domänen in Vpr mittels CD-Spektroskopie untersucht (Luo *et al.*, 1998):

Kurze, ca. 20 Aminosäure lange Peptide der C-terminalen Region von Vpr, welche das Motiv "HF/SRIG" enthalten, haben in einer Konzentration von 0.7 bis 3 micro-M zytotoxische Wirkungen gegenüber verschiedenen Hefe-Stämmen, wie zum Beispiel *Saccharomyces*

*cerevisiae*, *Candida albicans* und *Schizosaccharomyces pombe* (Macreadie *et al.*, 1996. 1997) auslöst. Eine erhöhte Konzentration von bivalenten Kationen. insbesondere Magnesium und Kalzium, verhindert die Aufnahme der Vpr-Peptide und dadurch deren toxische Effekte. Weiterführende Studien zeigten, daß ein C-terminales Vpr-Peptid (Positionen 71-82) die Membranpermeabilisierung, weiterhin eine Reduktion des Mitochondrienmembranpotentials und letztendlich den Zelltod von $CD4^+$ T-Zellen bewirkt (Macreadie *et al.*, 1997). Schließlich wurden ähnliche toxische Effekte ebenfalls für Gesamt-Vpr demonstriert (Arunagiri *et al.*, 1997). Dazu wurde das gleiche rekombinante Glutathione S-Transferase(GST)-Vpr-Fusionsprotein eingesetzt, welches zuvor für Ionenkanalstudien an Vpr verwendet wurde (Piller *et al.*, 1996). Jedoch berichten die Autoren ebenfalls über Probleme mit der Löslichkeit des rekombinanten Produktes in wässerigen Systemen.

Rekombinantes Vpr des Isolates HIV-$1_{NL4-3}$ wurde in Insektenzellen nach Infektion mit rekombinanten Baculoviren exprimiert (Levy *et al.*, 1995). Die Reinigung des Produktes erfolgte lediglich durch Immunaffinitätschromatographie an immobilisiertem polyklonalen Antiserum, welches gegen die N-terminale Domäne von Vpr gerichtet ist. Dazu wurden Zellkulturüberstände eingesetzt, da rekombinantes Vpr unspezifisch in das Kulturmedium sekretiert wird. Reinigungsstrategien für die Produktion größerer Mengen an rekombinanten Vpr wurden nicht beschrieben. In den meisten Fällen wurden von Autoren Vpr-haltige Zellkulturüberstände für biologische Tests verwendet. Dabei konnte gezeigt werden, daß rekombinantes Vpr die Virusreplikation in PBMC (peripheral blood mononuclear cells) und in verschiedenen latent infizierten Monozyten- und T-Zellinien aktiviert. Wesentliche Nachteile dieses Verfahrens sind:

- geringe Ausbeute und keine Möglichkeit zur Herstellung von mg-Mengen an hochreinem Produkt;
- rekombinantes Vpr wurde im Prozeß der Affinitätsreinigung mit Detergentien versetzt, wodurch Dialyse und Renaturierung notwendig wurden;
- Studien zu einer möglichen posttranslationalen Modifizierung von Vpr in Insektenzellen wurden nicht beschrieben;
- die Wirkung von rekombinanten Vpr in HIV-infizierten primären Monozyten / Makrophagen wurde nicht getestet.

Expression, Reinigung sowie biochemische Charakterisierung von rekombinanten Vpr wurden erstmals 1994 von Zhao und Mitarbeitern beschrieben. Dazu wurde die kodierende Sequenz des Vpr-Proteins des Isolates HIV-$1_{89.6}$ in *E. coli* als Fusionsprotein exprimiert. Zum Zweck der Reinigung und des Nachweises wurde in diesem Verfahren C-terminal eine 25 Aminosäuren lange Sequenz des heterologen FLAG-Epitopes fusioniert. Außer der Oligomerisierung wurde

über keine biologischen Aktivitäten des rekombinanten Produktes in dieser Arbeit berichtet. Wesentlicher Nachteil dieses Verfahrens ist die Tatsache. daß Vpr nicht in seiner authentischen Sequenz, sondern als Fusionsprotein exprimiert wird.

In einem weiteren Verfahren wurde Vpr des Isolates HIV-$1_{HXB2}$ in *E. coli* als GST-Fusionsprotein exprimiert (Piller *et al.*, 1996). Nach Affinitätschromatographie an Glutathione-Agarose wurde Vpr durch Thrombin-Spaltung vom Fusionsanteil befreit. Wesentlicher Nachteil dieses Verfahrens ist die Tatsache, daß Vpr nach Spaltung eine starke Tendenz zur Aggregation besitzt und nicht in wässeriger Lösung gehalten werden kann. So berichten zum Beispiel Arunagiri und Mitarbeiter (1997), daß mit diesem Verfahren hergestelltes rekombinantes Vpr nach Abspaltung des GST-Fusionsanteils nicht in Lösung gehalten werden kann, sondern nur durch Beibehaltung des heterologen Fusionsanteils Vpr in wässerigen Systemen getestet werden konnte.

In der Patentanmeldung WO 95/26361 (Azad, A.A., Macreadie, I.G., Arunagiri, C., 1995) werden biologisch aktive Peptidfragmente des Vpr-Proteins von HIV beschrieben; pharmazeutische Verbindungen, welche diese Peptide oder biologisch aktive Analoga davon enthalten; Antagonisten der Vpr-Peptide sowie pharmazeutische Verbindungen, welche diese Vpr-Antagonisten enthalten. Die chemische Synthese von Gesamt-Vpr-Protein spielt darin keine Rolle.

In der WO 96/07741 (Cohen, E.; Bergeron, D.; Checroune, F.; Yao, X.-J.; Pignac-Kobinger, G., 1996) werden chimere Moleküle unter Schutz gestellt, bestehend aus Vpr von HIV-1 und Vpx von HIV-2, welche spezifisch in HIV-1/HIV-2-Viruspartikel eingebaut werden können und dort die strukturelle Organisation und funktionelle Integrität von Virionen stören. Sie sind jedoch für den Einsatz zur Gentherapie von HIV-1/HIV-2-Infektionen ausgeschlossen.

In WO 96/08970 (Weiner, D.B.; Levy, D.N.; Refaeli, Y., 1996) werden Methoden zur Inhibierung der Zellteilung und der Lymphozyten-Aktivierung unter Anwendung von Vpr-Proteinen, Fragmenten von Vpr oder Gensequenzen von Vpr beschrieben. Die chemische Synthese von Vpr-Proteinen spielt darin keine Rolle.

Die Verwendung von *vpr* Genen im screening assay für anti-HIV-Arzneimittel wird in den US-Patenten 5721104 und 5639619 beschrieben, zur Bestimmung von HIV-2 in US 5580739, ein Vpr-Rezeptor-Protein in US 5780238.

Der Erfindung liegt die Aufgabe zugrunde. einen Syntheseweg für Vpr-Peptide im mg-Maßstab zu entwickeln, ihre Reinigung zu ermöglichen, und der Allgemeinheit das Endprodukt zur Verfügung zu stellen.

Die Aufgabe wurde erfindungsgemäß durch die Bereitstellung des Proteins sVpr1-96 sowie der Peptide

- ein 47 Aminosäuren langes N-terminales Peptid (sVpr1-47),
- ein 49 Aminosäuren langes C-terminales Peptid (sVpr48-96) und von Fragmenten dieser Peptide, zum Beispiel
- überlappende, etwa 15 Aminosäuren lange Peptide für die Epitop-Charakterisierung und isolelektrische Fokussierung
- etwa 20 Aminosäuren lange Peptide zur strukturellen und funktionellen Charakterisierung einzelner Domänen von Vpr, insbesondere die Peptide sVpr1-20 und sVpr21-40 gelöst:

sVpr1-96:

H-Met-Glu-Gln-Ala-Pro-Glu-Asp-Gln-Gly-Pro-Gln-Arg-Glu-Pro-Tyr-Asn-Glu-Trp-Thr-Leu-Glu-Leu-Leu-Glu-Glu-Leu-Lys-Ser-Glu-Ala-Val-Arg-His-Phe-Pro-Arg-Ile-Trp-Leu-His-Asn-Leu-Gly-Gln-His-Ile-Tyr-Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala-Gly-Val-Glu-Ala-Ile-Ile-Arg-Ile-Leu-Gln-Gln-Leu-leu-Phe-Ile-His-Phe-Arg-Ile-Gly-Cys-Arg-His-Ser-Arg-Ile-Gly-Val-Thr-Arg-Gln-Arg-Arg-Ala-Arg-Asn-Gly-Ala-Ser-Arg-Ser-OH

sVpr1–47:

H-Met-Glu-Gln-Ala-Pro-Glu-Asp-Gln-Gly-Pro-Gln-Arg-Glu-Pro-Tyr-Asn-Glu-Trp-Thr-Leu-Glu-Leu-Leu-Glu-Glu-Leu-Lys-Ser-Glu-Ala-Val-Arg-His-Phe-Pro-Arg-Ile-Trp-Leu-His-Asn-Leu-Gly-Gln-His-Ile-Tyr-$NH_2$

sVpr48-96:

Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala-Gly-Val-Glu-Ala-Ile-Ile-Arg-Ile-Leu-Gln-Gln-Leu-leu-Phe-Ile-His-Phe-Arg-Ile-Gly-Cys-Arg-His-Ser-Arg-Ile-Gly-Val-Thr-Arg-Gln-Arg-Arg-Ala-Arg-Asn-Gly-Ala-Ser-Arg-Ser-OH

sVpr1-20 als sVpr1–20($Asn^{5,10,14}$):

H-Met-Glu-Gln-Ala-Asn-Glu-Asp-Gln-Gly-Asn-Gln-Arg-Glu-Asn-Tyr-Asn-Glu-Trp-Thr-Leu-$NH_2$ und

sVpr21-40 als sVpr21–40($Asn^{35}$):

H-Glu-Leu-Leu-Glu-Glu-Leu-Lys-Ser-Glu-Ala-Val-Arg-His-Phe-Asn-Arg-Ile-Trp-Leu-His-$NH_2$

,

Fragmente dieser Peptide - mit etwa 15 Aminosäuren langen Peptiden

sVpr11-25:

Gln-Arg-Glu-Pro-Tyr-Asn-Glu-Trp-Thr-Leu-Glu-Leu-Leu-Glu-Glu-,

sVpr41-55:

Asn-Leu-Gly-Gln-His-Ile-Tyr-Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala,

sVpr46-60:

Ile-Tyr-Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala-Gly-Val-Glu-Ala-Ile-,

sVpr56-70:

Gly-Val-Glu-Ala-Ile-Ile-Arg-Ile-Leu-Gln-Gln-Leu-leu-Phe-Ile,

sVpr66–80:

Gln-Leu-leu-Phe-Ile-His-Phe-Arg-Ile-Gly-Cys-Arg-His-Ser-Arg,

sVpr76–96:

Cys-Arg-His-Ser-Arg-Ile-Gly-Val-Thr-Arg-Gln-Arg-Arg-Ala-Arg-Asn-Gly-Ala-Ser-Arg-Ser-OH,

Die Synthese der C-terminalen Vpr-Peptide erfolgte an einem Serin-Harz mit Hilfe eines Perkin-Elmer-Synthesizers. Alle N-terminalen Peptide wurden an einem Polystyren-Polyoxyethylen-Trägerharz synthetisiert. Der Aufbau der Peptide erfolgte mittels FMOC(Fluormethyloxycarbonyl)-Strategie unter Verwendung von Schutzgruppen. Nach Beendigung der Synthese erfolgte die Abspaltung der Schutzgruppen mittels eines Abspaltungsgemisches, bestehend aus 95% Trifluoressigsäure, der 3% Triisopropylsilan und je nach Peptid 2 bis 5 % Ethandithiol zugesetzt wurde. Das Harz wurde abgetrennt, die Reaktionslösung eingeengt und mit Heptan versetzt. Es wurde erneut eingeengt und das verbleibende Öl mit Diethylether digeriert. Das rohe Peptid wurde abgesaugt und anschließend aus Essigsäure lyophilisiert. Zur Reinigung wurden die Rohpeptide an einer präparativen HPLC-Anlage (High Pressure Liquid Chromatography) chromatographiert. Alle Peptide wurden an einer Kieselgelsäule mittels eines linearen Gradienten, bestehend aus TFA (Trifluoressigsäure) in Wasser und TFA in Acetonitril gereinigt. Die Eluate wurden eingeengt und lyophilisiert.

Überraschenderweise hat sich herausgestellt, daß die erfindungsgemäß hergestellten sVpr-Peptide nach dieser Reinigungsprozedur - im Unterschied zu den bislang beschriebenen rekombinanten oder synthetischen Produkten - wasserlöslich sind und selbst in hohen Konzentration von bis zu mM-Lösungen keiner Proteinaggregation unterliegen. Es konnte gezeigt werden, daß das Protein sVpr1-96 eine gefaltete Struktur annimmt, biologische Aktivitäten vergleichbar mit viralen Vpr hat und immunologisch reaktiv ist.

Erstmals wird die chemische Synthese des Vpr-Proteins und seiner Fragmente beschrieben, welcher der Aminosäuresequenz des Virusisolates HIV-$1_{NL4-3}$ entspricht.

Unter dem Begriff synthetische (s) Vpr-Peptide werden im Rahmen der vorliegenden Erfindungsbeschreibung die durch Festphasensynthese hergestellten Peptide verstanden, welche

die authentische Aminosäuresequenz des nativen Vpr-Proteins enthalten, so wie dieses durch das *vpr* Gen des molekularen Isolates HIV-$1_{NL4-3}$ kodiert wird.

Das Wesen der Erfindung liegt in einer Kombination bekannter Merkmale (Ausgangsstoffe, Syntheseharze, Synthesizer) und neuer Lösungswege – der erstmaligen chemischen Synthese dieser Verbindungen. der Synthesestrategie, der Wahl der spezifischen Schutzgruppen. dem erfindungsgemäßen Abspaltungsgemisch Trifluoressigsäure-Triisopropylsilan-Ethandithiol. dem Einsatz eines bestimmten Lösungsmittelgradienten (TFA-Wasser- : TFA-Acetonitril für die Reinigung - die sich gegenseitig beeinflussen und in ihrer neuen Gesamtwirkung einen Gebrauchsvorteil und den erstrebten Erfolg ergeben, der darin liegt, daß nunmehr neue synthetisch hergestellte sVpr-Peptide zur Verfügung stehen.

Die erfindungsgemäß hergestellten synthetischen Peptide zeichnen sich durch folgende Eigenschaften aus:

Sie haben eine extrem gute Löslichkeit in wässerigen Systemen, welche bis zu mM konzentrierte Peptid-Lösungen erlauben. Dies wiederum ist Voraussetzung für nachfolgende Strukturanalysen von Vpr mittels NMR(Nuclear Magnetic Resonance)-spektroskopischer und RKSA(Röntgenkristallstrukturanalyse)-Techniken.

Die Peptide lassen sich unter ökonomisch vertretbaren Bedingungen im mg-Maßstab herstellen und bis zu einem hohen Reinheitsgrad anreichern. Sie zeigen immunogene und biologische Eigenschaften, welche identisch sind mit denen von natürlichen Vpr-Proteinen. Sie lassen sich für vielfältige Gebiete der Grundlagenforschung sowie der angewandten Forschung auf dem Gebiet der HIV-Virologie einsetzen.

Die erfindungsgemäßen Peptide finden Verwendung in biologischen Assays, in der Strukturanalyse von Vpr und dessen Domänen, zur Erzeugung von Antikörpern gegen HIV-Peptidsequenzen, in antiviralen Reagenzien, zum Aufbau von Testsystemen zum Screenen von potentiellen Vpr-Antagonisten, bei der Etablierung von Zellkultur- und Tiermodellen, zur Untersuchung der Pathomechanismen von Vpr, für die *in vitro* Assemblierung von neuartigen Vektoren für den Einsatz bei Gentransfermethoden in der Gentherapie und zur Entwicklung von serologischen Testmethoden, insbesondere eines Vpr-Antigen-ELISA.

Die erfindungsgemäß hergestellten Produkte können für die Aufklärung der molekularen Struktur von Vpr mittels NMR- und CD-spektrokopischen Methoden sowie der Kristallisation und nachfolgender RKSA eingesetzt werden. Diese Informationen wiederum sind essentiell für das Verständnis der molekularen Wirkungsweise des Vpr-Proteins im HIV-1-Replikationszyklus und der damit verbundenen Pathomechanismen einer AIDS-Erkrankung sowie dem molekularen Design von potentiellen Vpr-Antagonisten.

Weiterhin können mit diesen Produkten *in vitro* Testsysteme dargestellt werden, welche das intensive Screening von potentiellen anti-Vpr-wirksamen Reagenzien erlauben. Darüber hinaus können sie für die Erzeugung und Testung von Vpr-spezifischen Antikörpern und für serologische Testverfahren angewendet werden.

Die Erfindung wird in der Peptidchemie, der virologischen Grundlagenforschung, der Strukturanalyse sowie der medizinischen Diagnostik angewendet.

Die Erfindung kann zur Herstellung von poly- und monoklonalen Vpr-spezifischen Antikörpern oder Antiseren, speziell zur Gewinnung von Epitop-differenten Vpr-spezifischen Antkörpern verwendet werden. Weitere Anwemdungsgebiete sind: serologische Testverfahren, als Vpr-Antigen(Ag)-ELISA, als Standard-Antigen für die Eichung von Vpr-Ag-ELISA-Techniken, Nachweis zur Konzentrationsbestimmung von viralem Vpr im Blut HIV-infizierter Individuen, Testsysteme zur Bestimmung von Vpr-Antagonisten, Komplementierung der Funktion von endogenen, viralen Vpr in Zellkulturen, die mit vpr-defizienten HIV-Mutanten infiziert sind, Komplementierung der Funktion von viralem Vpr in Kulturen von primären humanen Lymphozyten, die mit vpr-defizienten HIV-Mutanten infiziert sind und Komplementierung der Funktion von viralen Vpr in Kulturen von ausdifferenzierten primären humanen Monozyten / Makrophagen, die mit vpr-defizienten HIV-Mutanten infiziert sind.

Die Erfindung eignet sich außerdem zur Bestimmung von Reagenzien, die

a) die Wechselwirkung von Vpr mit zellulären Faktoren, wie zum Beispiel mit dem Glucocorticoid-Rezeptor, Transkriptionsfaktoren und anderen DNA-interagierenden Enzymen und Faktoren unterbinden;

b) die Transkriptions-aktivierende Wirkung von Vpr verhindern;

die Aktivität von Vpr auf die Wirkung von Steroidhormone regulieren, beeinflussen oder verhindern;

c) den Transport von Vpr allein oder im Verbund mit anderen Komponenten des HIV-Präintegrationskomplexes regulieren, beeinflussen oder verhindern;

den Einbau von Vpr in Viruspartikel während der HIV-Assemblierung regulieren, beeinflussen oder verhindern;

d) den Vpr-induzierten Zellzyklusarrest regulieren, beeinflussen oder verhindern

den Effekt von Vpr auf Zelldifferenzierung und Zellwachstum regulieren, beeinflussen oder verhindern

e) die zytotoxischen Effekte von Vpr regulieren, beeinflussen oder verhindern und

f) die Ionenkanalaktivität von Vpr regulieren, beeinflussen oder verhindern

Weiterhin ist die Verwendung für in vivo Testsysteme zur Bestimmung von Vpr-Antagonisten

möglich. Die Erfindung eignet sich auch für Tiermodellstudien. Ein weiterer Vorteil besteht darin, daß konzentrierter Peptid-Lösungen bereitgestellt werden können. So können spezifische Vpr-Antagonisten hergestellt werden. Ein weiteres Anwendungsgebiet ist die Reduktion der durch die N-terminale Domäne von Vpr induzierten Flexibilität von sVpr-Protein mittels strukturstabilisierenden Faktoren. Bei diesen Faktoren handelt es sich um die UBA2-Domäne des DNA-Reparaturproteins HHR23A, welches an Vpr bindet, Fab-Fragmente von Vpr-spezifischen Immunglobulinen oder virale Faktoren, insbesondere Komponenten des HIV-1 Gag-Polyproteinprecursurs Pr55Gag, welche im Prozess der Virus-Assemblierung mit Vpr in Verbindung treten, dem humanen Glucocorticoidrezeptor oder Bestandteile davon. Mit der Erfindung lassen sich eine in vitro Assemblierung von retroviralen Präintegrationskomplexen, in vitro oder in vivo applizierbaren Gentransfermethoden, Transfektionen, Integration in chromosomale und episomale Wirts-DNA oder andere Gentransfermethoden in eukaryotischen Zellen oder Gentransfers von in vitro hergestellter und/oder manipulierter Genfragmente in Zellen, Gewebe oder Organismen mit dem Zweck einer gentherapeutischen Applikation erreichen.

Sie soll anhand von Ausführungsbeispielen näher erläutert werden, ohne auf sie beschränkt zu sein.

**Ausführungsbeispiele**

Beispiel 1:

Synthese von Vpr-Peptiden - Allgemeine Vorschrift

Die Synthese der C-terminalen Vpr-Peptide erfolgte an einem Serin-Harz der Fa. Rapp Polymere Tübingen an einem ABI 433A Synthesizer (Perkin Elmer).

Alle N-terminalen Peptide wurden an einem Polystyren-polyoxyethylen-Trägerharz (TentaGel R-RAM-Harz der Fa. Rapp Polymere) synthetisiert.

Der Aufbau der Peptide erfolgte mittels FMOC(Fluormethyloxycarbonyl)-Strategie unter Verwendung nachfolgender Schutzgruppen: O-t.Butylester für Glu und Asp, OtBu-Ether für Serin. Tyrosin und Threonin. Boc (tert-Butoxycarbonyl-) für Lysin und Tryptophan. Trt (Trityl - Triphenylmethyl-) für Histidin, Glutamin und Asparagin sowie Pbf (2.2.4.6.7-pentamethyl-dihydrobenzofuran-5-sulfonyl-) für Arginin.

Nach Beendigung der Synthese erfolgte die Abspaltung der Schutzgruppen mittels eines Abspaltungsgemisches. bestehend aus 95% Trifluoressigsäure. der 3% Triisopropylsilan und je nach Peptid 2 bis 5 % Ethandithiol zugesetzt wurde. Das Harz wurde abgetrennt. die Reaktionslösung eingeengt und mit Heptan versetzt. Es wurde erneut eingeengt und das verbleibende Öl mit Diethylether digeriert. Das rohe Peptid wurde abgesaugt und anschließend aus 10%iger Essigsäure lyophilisiert.

Beispiel 2:

Reinigung der Peptide - Allgemeine Vorschrift

Zur Reinigung wurden jeweils 100 mg Rohpeptid an einer präparativen HPLC-Anlage (Shimadzu LC-8 Anlage) chromatographiert. Alle Peptide wurden an einer Kieselgelsäule (300 x 400 mm Vydac-RP18-Säule, Korngröße 15 - 20 µM) mittels eines linearen Gradienten, bestehend aus A = 1% TFA (Trifluoressigsäure) in Wasser und B = 0,1% TFA in 80%igem Acetonitril mit einem Fluss von 100 ml / min gereinigt. Die Eluate wurden eingeengt und lyophilisiert.

Beispiel 3:

sVpr1-96

Das Peptid wurde an einem TentaGel S-AC-Harz (0,20 mmol/Gramm) an einem ABI 433 aufgebaut. Am Schluß der Synthese wurde die FMOC-Schutzgruppe abgespalten. das Harz nacheinander mit Dimethylformamid und Methylenchlorid gewaschen und getrocknet. Das Peptid wurde dann in der eingangs beschriebenen Weise vom Harz abgespalten und anschließend gereinigt.

Molmasse: 11378 gef. 11381

H-Met-Glu-Gln-Ala-Pro-Glu-Asp-Gln-Gly-Pro-Gln-Arg-Glu-Pro-Tyr-Asn-Glu-Trp-Thr-Leu-Glu-Leu-Leu-Glu-Glu-Leu-Lys-Ser-Glu-Ala-Val-Arg-His-Phe-Pro-Arg-Ile-Trp-Leu-His-Asn-Leu-Gly-Gln-His-Ile-Tyr-Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala-Gly-Val-Glu-Ala-Ile-Ile-Arg-Ile-Leu-Gln-Gln-Leu-leu-Phe-Ile-His-Phe-Arg-Ile-Gly-Cys-Arg-His-Ser-Arg-Ile-Gly-Val-Thr-Arg-Gln-Arg-Arg-Ala-Arg-Asn-Gly-Ala-Ser-Arg-Ser-OH

Figur 1: sVpr1-96 - Direkte Auftrennung im SDS-PAGE (A)
Immunpräzipitation vor SDS-PAGE (B)

Figur 2: sVpr1-96 - Präparative Reinigung des Rohpeptids - HPLC-Chromatogramm

Figur 3: sVpr1-96 - Massenspektrum (% Int. und Molmasse)

Beispiel 4:

sVpr1-47

Analog zu Beispielen 1 bis 3.

Molmasse: 5728 gef. 5728.8

H-Met-Glu-Gln-Ala-Pro-Glu-Asp-Gln-Gly-Pro-Gln-Arg-Glu-Pro-Tyr-Asn-Glu-Trp-Thr-Leu-Glu-Leu-Leu-Glu-Glu-Leu-Lys-Ser-Glu-Ala-Val-Arg-His-Phe-Pro-Arg-Ile-Trp-Leu-His-Asn-Leu-Gly-Gln-His-

Ile-Tyr-$NH_2$

Figur 4: sVpr1-47 - Massenspektrum (% Int. und Molmasse)

Beispiel 5:

sVpr48-96

Analog zu Beispielen 1 bis 3.

Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala-Gly-Val-Glu-Ala-Ile-Ile-Arg-Ile-Leu-Gln-Gln-Leu-leu-Phe-Ile-His-Phe-Arg-Ile-Gly-Cys-Arg-His-Ser-Arg-Ile-Gly-Val-Thr-Arg-Gln-Arg-Arg-Ala-Arg-Asn-Gly-Ala-Ser-Arg-Ser-OH

Beispiel 6:

sVpr1-20

Analog zu Beispielen 1 bis 3.

H-Met-Glu-Gln-Ala-Pro-Glu-Asp-Gln-Gly-Pro-Gln-Arg-Glu-Pro-Tyr-Asn-Glu-Trp-Thr-Leu-

$NH_2$

Figur 5: sVpr1-20 - Massenspektrum (%Int. 10% =111 mV[sum= 9505 mV] Profiles 1-85 Unsmoothed und Molmasse)

Beispiel 7:

sVpr1-20($Asn^{5,10,14}$)

Analog zu Beispielen 1 bis 3.

H-Met-Glu-Gln-Ala-Pro-Glu-Asp-Gln-Gly-Pro-Gln-Arg-Glu-Pro-Tyr-Asn-Glu-Trp-Thr-Leu-

$NH_2$

Beispiel 8:

sVpr21-40

Analog zu Beispielen 1 bis 3.

Wildtyp-Sequenz

H-Glu-Leu-Leu-Glu-Glu-Leu-Lys-Ser-Glu-Ala-Val-Arg-His-Phe-Asn-Arg-Ile-Trp-Leu-His-$NH_2$

Figur 6: sVpr21-40 - Massenspektrum (%Int. 10% =335 mV[sum= 28541 mV] Profiles 1-85 Unsmoothed und Molmasse)

Beispiel 9:

sVpr21-40($Asn^{35}$)

Analog zu Beispielen 1 bis 3.

H-Glu-Leu-Leu-Glu-Glu-Leu-Lys-Ser-Glu-Ala-Val-Arg-His-Phe-Asn-Arg-Ile-Trp-Leu-His-$NH_2$

Beispiel 10:

sVpr11-25:

Analog zu Beispielen 1 bis 3.

Gln-Arg-Glu-Pro-Tyr-Asn-Glu-Trp-Thr-Leu-Glu-Leu-Leu-Glu-Glu-

Beispiel 11:

sVpr41-55:

Analog zu Beispielen 1 bis 3.

Asn-Leu-Gly-Gln-His-Ile-Tyr-Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala

Beispiel 12:

sVpr46-60:

Analog zu Beispielen 1 bis 3.

Ile-Tyr-Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala-Gly-Val-Glu-Ala-Ile-

Beispiel 13:

sVpr56-70:

Analog zu Beispielen 1 bis 3.

Gly-Val-Glu-Ala-Ile-Ile-Arg-Ile-Leu-Gln-Gln-Leu-leu-Phe-Ile

Beispiel 14:

sVpr66–80:

Analog zu Beispielen 1 bis 3.

Gln-Leu-Leu-Phe-Ile-His-Phe-Arg-Ile-Gly-Cys-Arg-His-Ser-Arg

Beispiel 15:

sVpr76–96

Analog zu Beispielen 1 bis 3.

Cys-Arg-His-Ser-Arg-Ile-Gly-Val-Thr-Arg-Gln-Arg-Arg-Ala-Arg-Asn-Gly-Ala-Ser-Arg-Ser-OH

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**Legende zu den Figuren**

Figur 1: Struktur- und Funktionsdomänen in Vpr

Folgende Primär- und Sekundär-Strukturelemente in Vpr sind der Aminosäuresequenz des Proteins Vpr von HIV-1NL4-3 zugeordnet: negativ geladener N-Terminus (Markierung (1), Positionen 1-17); Helix alpha-1 (Markierung (2), Positionen 18-37); eine nicht näher definierte Region (Markierung (3), Positionen 38-51); Helix alpha-2 (Markierung (4), Positionen 51-76); basischer C-Terminus (Markierung (8), Positionen 77-96). Überlappend dazu sind weitere Bereiche angezeigt: eine Leuzin- und Isoleuzin-reiche Region, welche auch als Leuzin-Zipper-ähnlich oder auch "LR-Domäne" bezeichnet wird (Markierung (5), Positionen 60-80); eine Region, welche das sich wiederholende Motiv "HF/SRIG" enthält (Markierung (6), Positionen 71-82); den vermutlichen Transmembrananker von Vpr, welcher notwendig für die Ionenkanalaktivität von Vpr ist (Markierung (7), Positionen 52-79).

Figur 2: Immunologische Reaktivität von polyklonalen Antikörpern spezifisch für sVpr1-96 im Westernblot und Immunpräzipitation

Serum von Kaninchen immunisiert mit sVpr1-96, R-96, wurde in Westernblot (A) und Immunpräzipitation (B) getestet. Eine Verdünnungsreihe von 0.01 bis 10 ng sVpr1-96 wurde im SDS-PAGE (12.5% Acryl aide Gel) aufgetrennt (A). Eine ähnliche Verdünnungsreihe an sVpr1-96 wurde mit humanen Serum versetzt, und aus diesem Gemisch wurde mittels dem Serum R-96 das Peptid sVpr1-96 durch Immunpräzipitation isoliert, und nachfolgend ebenfalls im SDS-PAGE aufgetrennt (B). Nach Elektrotransfer auf PVDF-Membranen wurde sVpr1-96 mittels R-96 Antikörpern sowie anschließender Bindung von 125I-Protein G detektiert. Das Autoradiogramm einer 2-Tage-Exposition ist in (A) und (B) dargestellt. Die Positionen von Molekulargewichtsstandardproteinen sind auf der linken Seite, sowie die Positionen von unspezifischen Reaktion mit der schweren (hc) und leichten Kette (lc) der zur Immunpräzipitation eingesetzten Immunglobuline ist auf der rechten Seite angezeigt.

Figur 3: sVpr1-96 aktiviert Virusreplikation und erhöht Zahl lebender Zellen in Kulturen von humanen PBMC

Kulturen von PHA- und IL-2-aktivierte PBMCs wurden mit gleichen infektiösen Dosen folgender Virusstocks infiziert: HIV-1NL4-3 (A.B.C), NL4-3(AD8) (D) sowie der vpu-defizienten Mutante NL(AD8)-UDEL1 (E) und der vpr-defizienten Mutante NL(AD8)deltaR (F). Während des Infektionsexperimentes wurden die Kulturen in Gegenwart von 10 nM sVpr1-96 oder 10 nM des Kontrollpeptides Vpu32-81 kultiviert. Die Virusfreisetzung ist als Profil der Virus-assoziierten RT-Aktivität im Zellkulturüberstand dargestellt (A,C,D,E,F). (B) zeigt die Zahl der lebenden Zellen im Experiment von (A).

Figur 4: sVpr1-96 aktiviert die Replikationskompetenz von vpr-defizienten HIV-1 Mutanten in Kulturen von primären humanen Monozyten/Makrophagen isoliert von verschiedenen Donoren Parallele Kulturen von ausdifferenzierten MDM-Isolaten gewonnen, von drei verschiedenen Donoren, wurden mit gleichen infektiösen Dosen von gereinigten Virusstocks des Makrophagen-tropen Virus NL4-3(AD8) sowie dessen vpr-defizienten Mutante NL(AD8)deltaR infiziert. Die Virusproduktion wurde über einen Zeitraum von etwa zwei Monaten verfolgt und als Virus-assozierte RT-Aktivität gegen die Zeit aufgetragen.

Figur 5: 2D 1H TOCSY Spektrum
(Mischungszeit = 110 ms) einer 2 mM-Lösung of sVpr1-96 in 1:1 (V/V) TFE-d2/H2= bei 300°K.

Die Ordinate und Abzisse zeigen die entsprechenden 1D 1H Spektren. Vergrößerungen der Regionen A, B und C werden in Figur 6 gezeigt.

Figur 6:
Vergrößerte Regionen der 2D TOCSY Spektren, dargestellt in Figur 5, welche den Wechselwirkungen zwischen den Protonen H-7 and H-2 von Tryptophanresten (A); H-2 und H-4 von Histidinresten (B), und epsilon-H und alpha-H von Argininresten (C) entsprechen.

Figur 7: sVpr1-96 - Chromatogramm und Massenspektrum

Figur 8: sVpr1-47 - Massenspektrum

Figur 9: sVpr1-20 - Massenspektrum

Figur 10: sVpr21-40 - Massenspektrum

**Patentansprüche**

1. Synthetische Peptide des regulatorischen Virusproteins R (Vpr) des Humanen Immundefizienzvirus Typ 1 (HIV-1).

2. Peptide nach Anspruch 1, dadurch gekennzeichnet, daß es sich um

2.1. ein 96 Aminosäuren langes Vpr-Protein (sVpr1-96)

2.2. ein 47 Aminosäuren langes N-terminales Peptid (sVpr1-47)

2.3. ein 49 Aminosäuren langes C-terminales Peptid (sVpr48-96) sowie

2.4. Fragmente dieser Peptide, zum Beispiel

2.4.1. überlappende, etwa 15 Aminosäuren lange Peptide für die Epitop-Charakterisierung und isolelektrische Fokussierung

2.4.2. etwa 20 Aminosäuren lange Peptide zur strukturellen und funktionellen Charakterisierung einzelner Domänen von Vpr, insbesondere

2.4.2.1. die Peptide sVpr1-20 und

2.4.2.2. sVpr21-40

handelt.

3. Peptide nach Ansprüchen 1 und 2, dadurch gekennzeichnet, daß es sich

3.1. bei dem 96 Aminosäuren langen Vpr-Protein um

sVpr1-96

H-Met-Glu-Gln-Ala-Pro-Glu-Asp-Gln-Gly-Pro-Gln-Arg-Glu-Pro-Tyr-Asn-Glu-Trp-Thr-Leu-Glu-Leu-Leu-Glu-Glu-Leu-Lys-Ser-Glu-Ala-Val-Arg-His-Phe-Pro-Arg-Ile-Trp-Leu-His-Asn-Leu-Gly-Gln-His-Ile-Tyr-Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala-Gly-Val-Glu-Ala-Ile-Ile-Arg-Ile-Leu-Gln-Gln-Leu-leu-Phe-Ile-His-Phe-Arg-Ile-Gly-Cys-Arg-His-Ser-Arg-Ile-Gly-Val-Thr-Arg-Gln-Arg-Arg-Ala-Arg-Asn-Gly-Ala-Ser-Arg-Ser-OH

3.2. bei dem 47 Aminosäuren langen N-terminalen Peptid um

sVpr1–47

H-Met-Glu-Gln-Ala-Pro-Glu-Asp-Gln-Gly-Pro-Gln-Arg-Glu-Pro-Tyr-Asn-Glu-Trp-Thr-Leu-Glu-Leu-Leu-Glu-Glu-Leu-Lys-Ser-Glu-Ala-Val-Arg-His-Phe-Pro-Arg-Ile-Trp-Leu-His-Asn-Leu-Gly-Gln-His-Ile-Tyr-$NH_2$

3.3. bei dem 49 Aminosäuren langen C-terminalen Peptid um
sVpr48-96
Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala-Gly-Val-Glu-Ala-Ile-Ile-Arg-Ile-Leu-Gln-Gln-Leu-leu-Phe-Ile-His-Phe-Arg-Ile-Gly-Cys-Arg-His-Ser-Arg-Ile-Gly-Val-Thr-Arg-Gln-Arg-Arg-Ala-Arg-Asn-Gly-Ala-Ser-Arg-Ser-OH

3.4. bei den Fragmenten dieser Peptide um die etwa 15 Aminosäuren lange Peptide
3.4.1. sVpr11-25
Gln-Arg-Glu-Pro-Tyr-Asn-Glu-Trp-Thr-Leu-Glu-Leu-Leu-Glu-Glu-
3.4.2. sVpr41-55
Asn-Leu-Gly-Gln-His-Ile-Tyr-Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala
3.4.3. sVpr46-60
Ile-Tyr-Glu-Thr-Tyr-Gly-Asp-Thr-Trp-Ala-Gly-Val-Glu-Ala-Ile-
3.4.4. sVpr56-70
Gly-Val-Glu-Ala-Ile-Ile-Arg-Ile-Leu-Gln-Gln-Leu-leu-Phe-Ile

3.5. bei den etwa 20 Aminosäuren langen Peptiden um
3.5.1. die Peptide sVpr1-20 als
sVpr1–20($Asn^{5,10,14}$)
H-Met-Glu-Gln-Ala-Asn-Glu-Asp-Gln-Gly-Asn-Gln-Arg-Glu-Asn-Tyr-Asn-Glu-Trp-Thr-Leu-$NH_2$
und
3.5.2. sVpr21-40 als
sVpr 21–40($Asn^{35}$)
H-Glu-Leu-Leu-Glu-Glu-Leu-Lys-Ser-Glu-Ala-Val-Arg-His-Phe-Asn-Arg-Ile-Trp-Leu-His-$NH_2$
handelt.

4. Verfahren zur Herstellung von neuen synthetischen Peptiden des regulatorischen Virusproteins R (Vpr) des Humanen Immundefizienzvirus Typ 1 (HIV-1) nach den Ansprüchen 1 bis 3, dadurch gekennzeichnet, daß die Synthese der C-terminalen Vpr-Peptide an einem Serin-Harz mit Hilfe eines Perkin-Elmer-Synthesizers erfolgt, alle N-terminalen Peptide an einem Polystyren-Polyoxyethylen-Trägerharz synthetisiert werden und der Aufbau der Peptide mittels FMOC-Strategie unter Verwendung von Schutzgruppen erfolgt.

5. Verfahren nach Anspruch 4, dadurch gekennzeichnet, daß nach Beendigung der Synthese die Abspaltung der Schutzgruppen mittels eines Abspaltungsgemisches, bestehend aus 95% Trifluoressigsäure, der 3% Triisopropylsilan und je nach Peptid 2 bis 5 % Ethandithiol zugesetzt wurde, erfolgt und das Harz abgetrennt wird.

6. Verfahren nach den Ansprüchen 4 und 5, dadurch gekennzeichnet, daß die Rohpeptide an einer präparativen HPLC-Anlage chromatographiert und die Peptide an einer Kieselgelsäule mittels eines linearen Gradienten, bestehend aus TFA (Trifluoressigsäure) in Wasser und TFA in Acetonitril, gereinigt werden.

7. Verwendung von synthetischen (s) Peptiden des regulatorischen Virusproteins R (Vpr) Humaner Immundefizienzviren (HIV) zu therapeutischen und/oder diagnostischen Zwecken.

8. Verwendung nach Anspruch 7

8.1. in biologischen Assays

8.1.1. zur Entwicklung von serologischen Testmethoden

8.1.2. zur Entwicklung eines Vpr-Antigen-ELISA

8.2. zur Erzeugung von Antikörpern gegen HIV-Peptidsequenzen

8.3. in antiviralen Reagenzien

8.4. zum Aufbau von Testsystemen zum Screenen von potentiellen Vpr-Antagonisten

8.5. bei der Etablierung von Zellkultur- und Tiermodellen zur Untersuchung der Pathomechanismen von Vpr

8.6. in der Strukturanalyse von Vpr und dessen Domänen oder

8.7. bei der *in vitro* Assemblierung von neuartigen Vektoren für den Einsatz bei Gentransfermethoden in der Gentherapie.

9. Verwendung nach Anspruch 7 und 8, dadurch gekennzeichnet, daß es sich um sVpr-Proteine handelt, in denen die N-terminale Domäne der sVpr-Proteine in einem, mehreren oder allen vier Prolin-Reste mutiert ist.

10. Verwendung nach Anspruch 7 bis 9 zur Herstellung von poly- und monoklonalen Vpr-spezifischen Antikörpern oder Antiseren.

11. Verwendung nach Anspruch 7 bis 10 zur Gewinnung von Epitop-differenten Vpr-

spezifischen Antkörpern.

12. Verwendung von Antikörpern nach Anspruch 7 bis 11 in serologischen Testverfahren.

13. Verwendung nach Anspruch 7 bis 12 in einem Vpr-Antigen(Ag)-ELISA.

14. Verwendung von sVpr-Proteinen nach Anspruch 7 bis 13 als Standard-Antigen für die Eichung von Vpr-Ag-ELISA-Techniken.

15. Verwendung nach Anspruch 7 und 8 zum Nachweis und zur Konzentrationsbestimmung von viralem Vpr im Blut HIV-infizierter Individuen.

16. Verwendung von sVpr-Proteinen nach Anspruch 7 und 8 für in vitro Testsysteme zur Bestimmung von Vpr-Antagonisten.

17. Verwendung nach Anspruch 7, 8 zur Komplementierung der Funktion von endogenen, viralen Vpr in Zellkulturen, die mit vpr-defizienten HIV-Mutanten infiziert sind.

18. Verwendung nach Anspruch 7, 8 und 17 zur Komplementierung der Funktion von viralem Vpr in Kulturen von primären humanen Lymphozyten, die mit vpr-defizienten HIV-Mutanten infiziert sind.

19. Verwendung nach Anspruch 7, 8, 17 und 18 zur Komplementierung der Funktion von viralen Vpr in Kulturen von ausdifferenzierten primären humanen Monozyten / Makrophagen, die mit vpr-defizienten HIV-Mutanten infiziert sind.

20. Verwendung nach Anspruch 7 bis 19 zur Bestimmung von Reagenzien, die

a) die Wechselwirkung von Vpr mit zellulären Faktoren, wie zum Beispiel mit dem Glucocorticoid-Rezeptor, Transkriptionsfaktoren und anderen DNA-interagierenden Enzymen und Faktoren unterbinden;
b) die Transkriptions-aktivierende Wirkung von Vpr verhindern;
die Aktivität von Vpr auf die Wirkung von Steroidhormone regulieren, beeinflussen oder verhindern;

c) den Transport von Vpr allein oder im Verbund mit anderen Komponenten des HIV-Präintegrationskomplexes regulieren, beeinflussen oder verhindern;
den Einbau von Vpr in Viruspartikel während der HIV-Assemblierung regulieren, beeinflussen oder verhindern;
d) den Vpr-induzierten Zellzyklusarrest regulieren, beeinflussen oder verhindern
den Effekt von Vpr auf Zelldifferenzierung und Zellwachstum regulieren, beeinflussen oder verhindern
e) die zytotoxischen Effekte von Vpr regulieren, beeinflussen oder verhindern
f) die Ionenkanalaktivität von Vpr regulieren, beeinflussen oder verhindern

21. Verwendung von sVpr-Proteinen nach Anspruch 7 und 8 für in vivo Testsysteme zur Bestimmung von Vpr-Antagonisten.

22. Verwendung von sVpr-Proteinen nach Anspruch 7 und 8 in Tiermodellstudien zur Bestimmung von Funktionen nach Anspruch 20.

23. Verwendung von sVpr-Proteinen nach Anspruch 7 und 8 zur Herstellung konzentrierter Peptid-Lösungen.

24. Verwendung von sVpr-Proteinen nach Anspruch 7, 8 und 23 zur Herstellung spezifischer Vpr-Antagonisten.

25. Verwendung von sVpr-Proteinen nach Anspruch 7, 8, 21 und 24 zur Reduktion der durch die N-terminale Domäne von Vpr induzierten Flexibilität von sVpr-Protein mittels strukturstabilisierenden Faktoren.

26. Verwendung nach Anspruch 25, dadurch gekennzeichnet, daß es sich bei den strukturstabilisierenden Faktoren um
a) die UBA2-Domäne des DNA-Reparaturproteins HHR23A, welches an Vpr bindet,
b) Fab-Fragmente von Vpr-spezifischen Immunglobulinen oder
c) virale Faktoren, insbesondere Komponenten des HIV-1 Gag-Polyproteinprecursurs Pr55Gag, welche im Prozess der Virus-Assemblierung mit Vpr in Verbindung treten oder
d) dem humanen Glucocorticoidrezeptor oder Bestandteile davon
handelt.

27. Verwendung von sVpr-Proteinen nach Anspruch 7 für in vitro Assemblierung von retroviralen Präintegrationskomplexen.

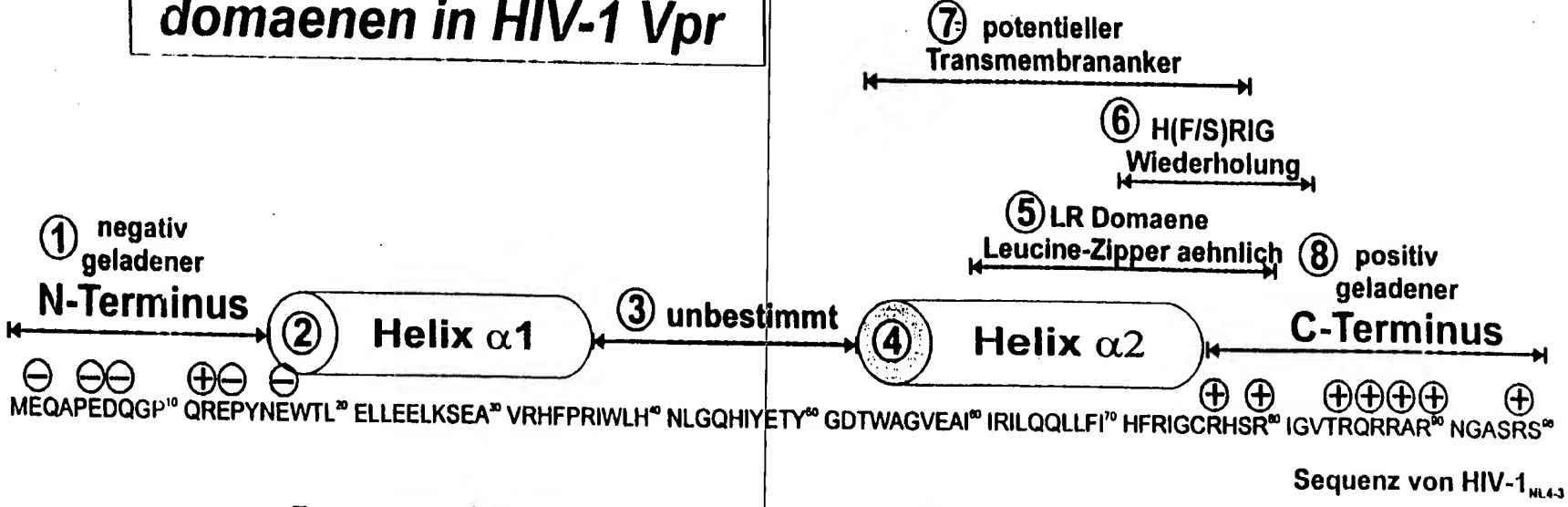
28. Verwendung von sVpr-Proteinen nach Anspruch 7, 8 und 27 in in vitro oder in vivo applizierbaren Gentransfermethoden.

29. Verwendung von sVpr-Proteinen nach Anspruch 7, 8 und 28 für Transfektionen, Integration in chromosomale und episomale Wirts-DNA oder andere Gentransfermethoden in eukaryotischen Zellen.

30. Verwendung von sVpr-Proteinen nach Anspruch 7, 8 und 28 für Gentransfers von in vitro hergestellter und/oder manipulierter Genfragmente in Zellen, Gewebe oder Organismen mit dem Zweck einer gentherapeutischen Applikation.

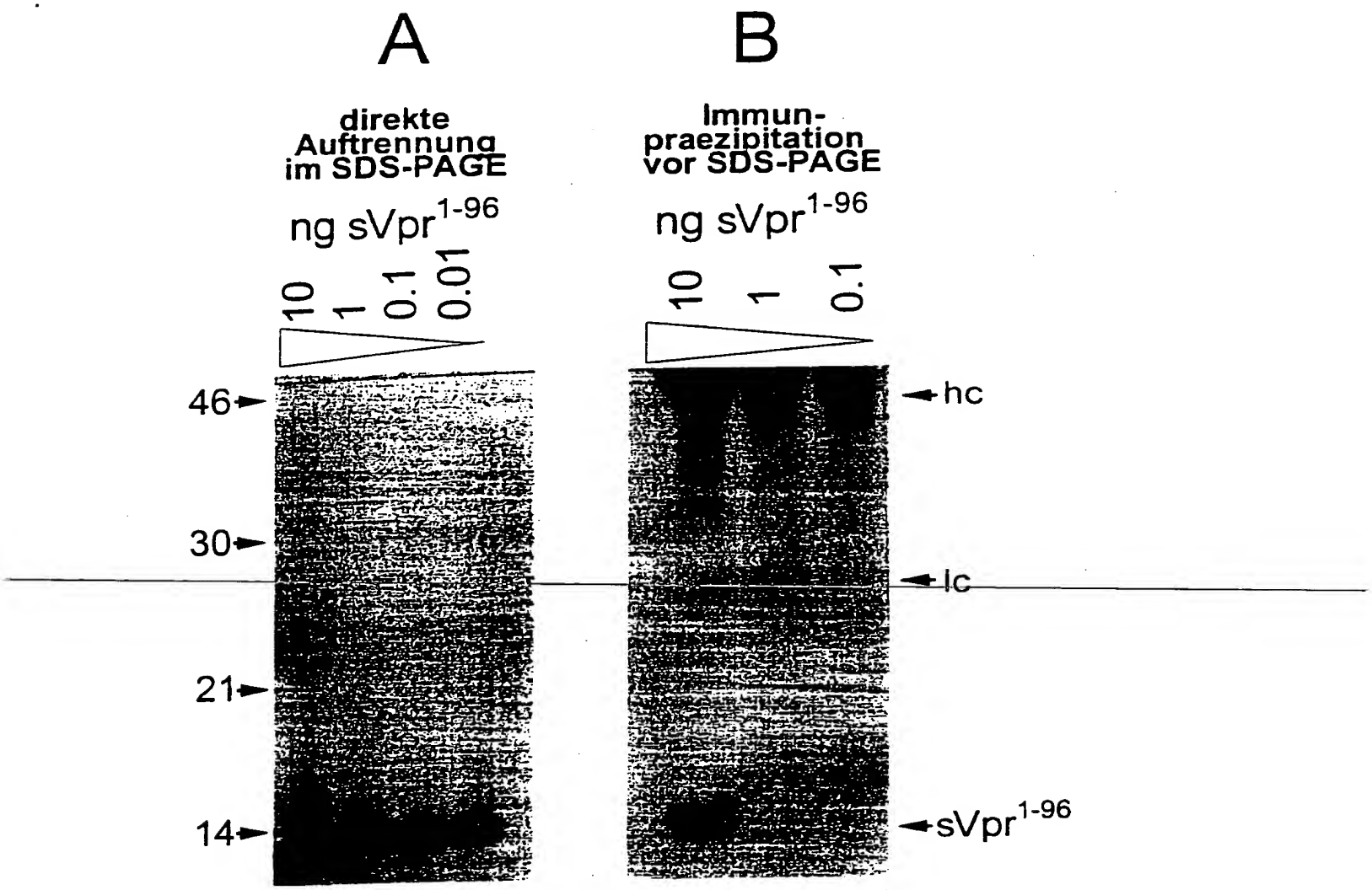
Figur 1

# Struktur- und Funktions-domaenen in HIV-1 Vpr

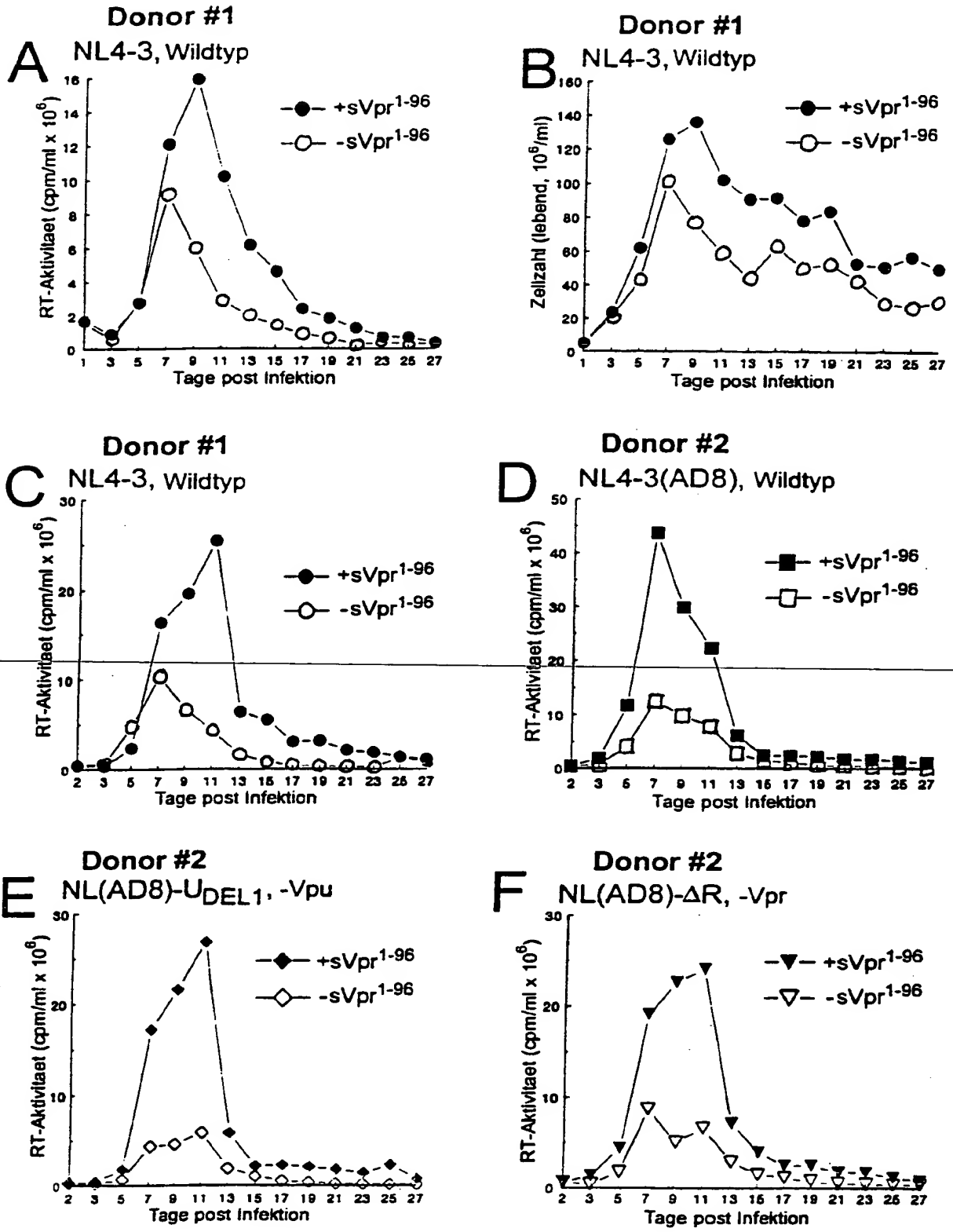



| Domaene | Funktionen |
|---|---|
| ② ④ | Einbau von Vpr in Viruspartikel |
| ② ④ ⑤ | Transport in Zellkern |
| ② ② ④ | Zellzyklus-Arrest/Zelldifferenzierung |
| ① ② ③ | Oligomerisierung |
| ⑥ ⑧ | Zytotoxizitaet und Induktion von Apoptosis |
| ④ ⑧ | Ionenkanalaktivitaet |
| ⑤ ⑧ | Transkriptionsaktivierung & Interaktion mit Sp1 |
| ④ ⑤ | Regulation des Glucocortikoidrezeptors |

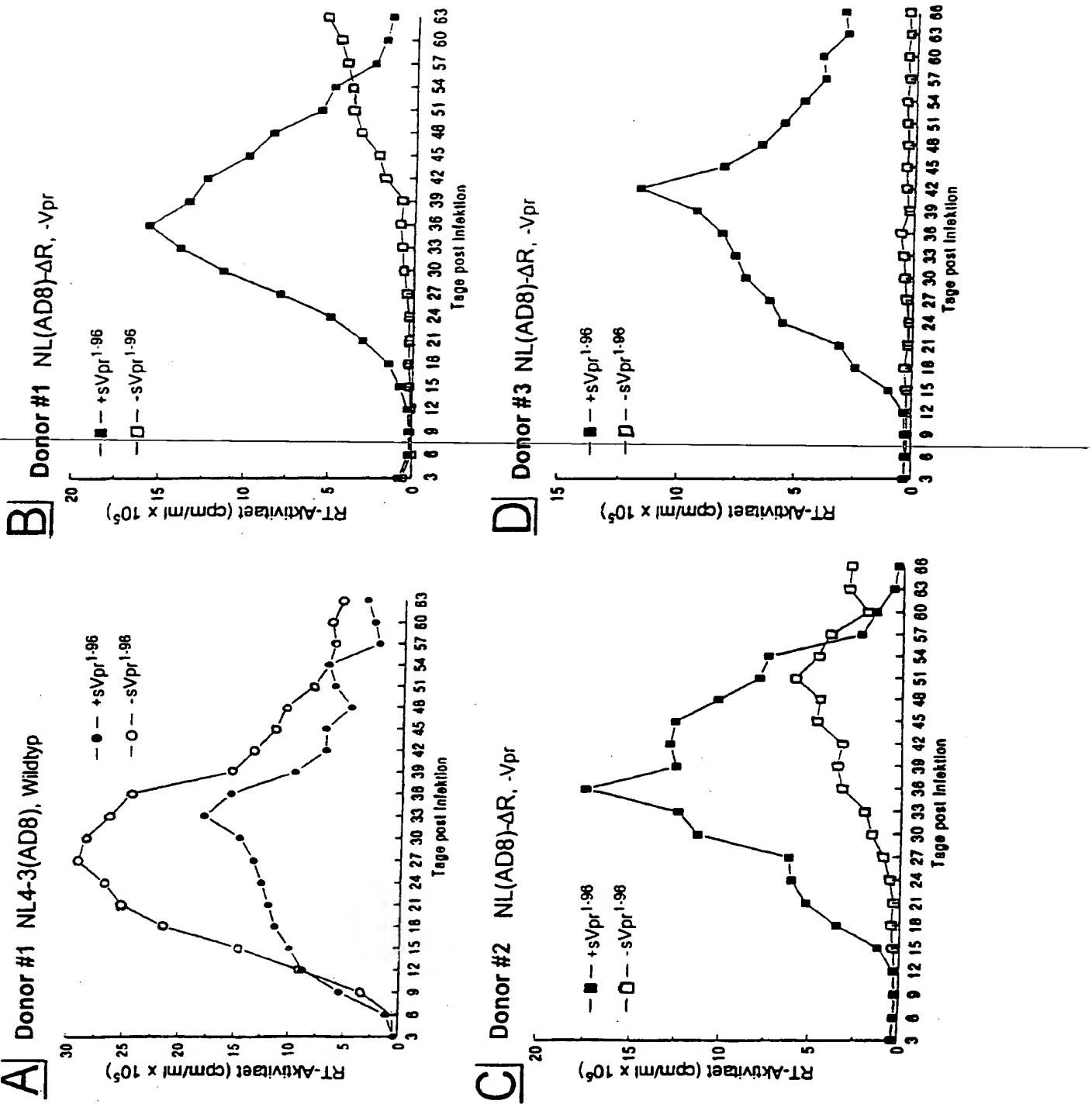
Figur 2


A
B
direkte Auftrennung im SDS-PAGE
Immun-praezipitation vor SDS-PAGE
ng sVpr1-96
10
1
0.1
0.01
ng sVpr1-96
10
1
0.1
46
30
21
14
hc
lc
sVpr1-96

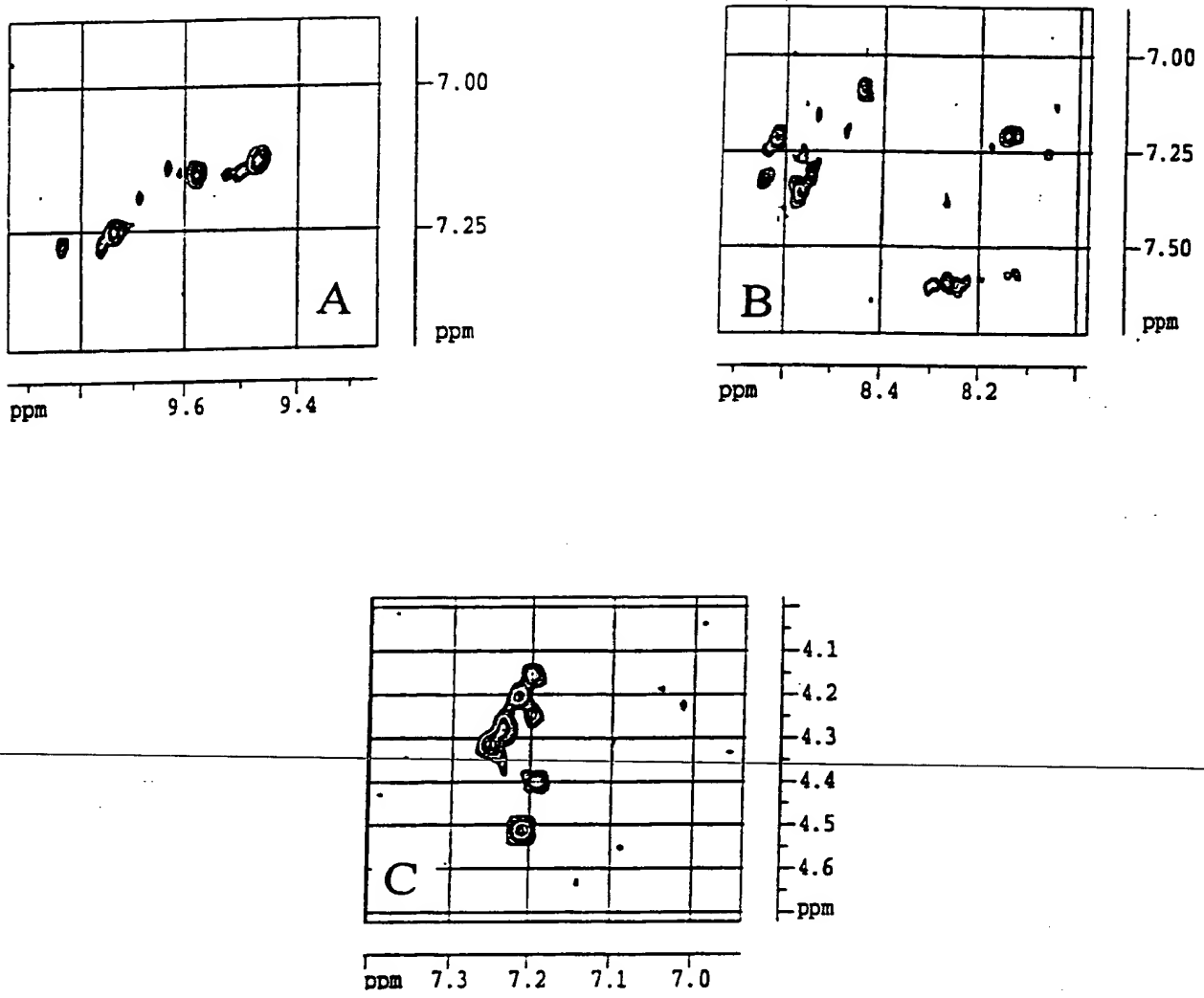
Figur 3


Donor #1
A NL4-3, Wildtyp
RT-Aktivitaet (cpm/ml x 10^6)
+sVpr1-96
-sVpr1-96
Tage post Infektion
Donor #1
B NL4-3, Wildtyp
Zellzahl (lebend, 10^6/ml)
+sVpr1-96
-sVpr1-96
Tage post Infektion
Donor #1
C NL4-3, Wildtyp
RT-Aktivitaet (cpm/ml x 10^6)
+sVpr1-96
-sVpr1-96
Tage post Infektion
Donor #2
D NL4-3(AD8), Wildtyp
RT-Aktivitaet (cpm/ml x 10^6)
+sVpr1-96
-sVpr1-96
Tage post Infektion
Donor #2
E NL(AD8)-U_DEL1, -Vpu
RT-Aktivitaet (cpm/ml x 10^6)
+sVpr1-96
-sVpr1-96
Tage post Infektion
Donor #2
F NL(AD8)-ΔR, -Vpr
RT-Aktivitaet (cpm/ml x 10^6)
+sVpr1-96
-sVpr1-96
Tage post Infektion

Figur 4


A
Donor #1 NL4-3(AD8), Wildtyp
+sVpr1-96
-sVpr1-96
RT-Aktivität (cpm/ml x 10⁵)
Tage post Infektion
B
Donor #1 NL(AD8)-ΔR, -Vpr
+sVpr1-96
-sVpr1-96
RT-Aktivität (cpm/ml x 10⁵)
Tage post Infektion
C
Donor #2 NL(AD8)-ΔR, -Vpr
+sVpr1-96
-sVpr1-96
RT-Aktivität (cpm/ml x 10⁵)
Tage post Infektion
D
Donor #3 NL(AD8)-ΔR, -Vpr
+sVpr1-96
-sVpr1-96
RT-Aktivität (cpm/ml x 10⁵)
Tage post Infektion

Figur 5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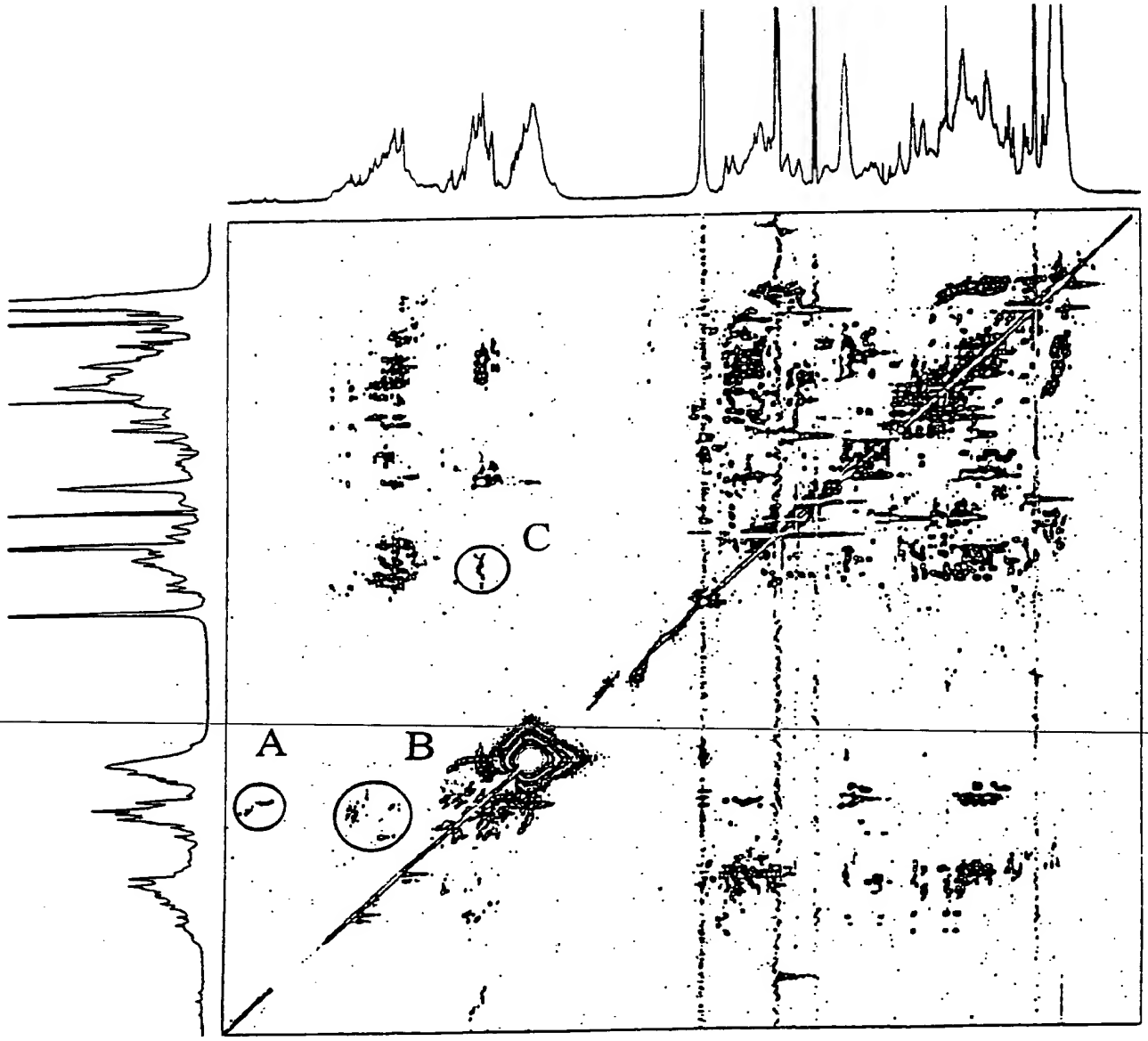
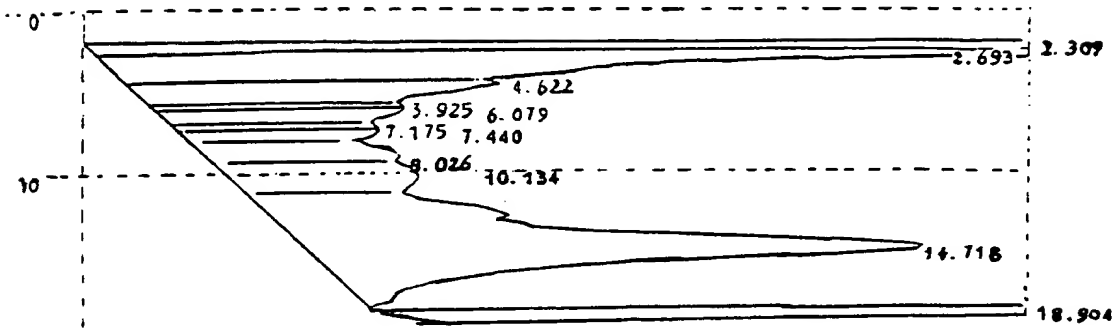
Figur 6



Figur 7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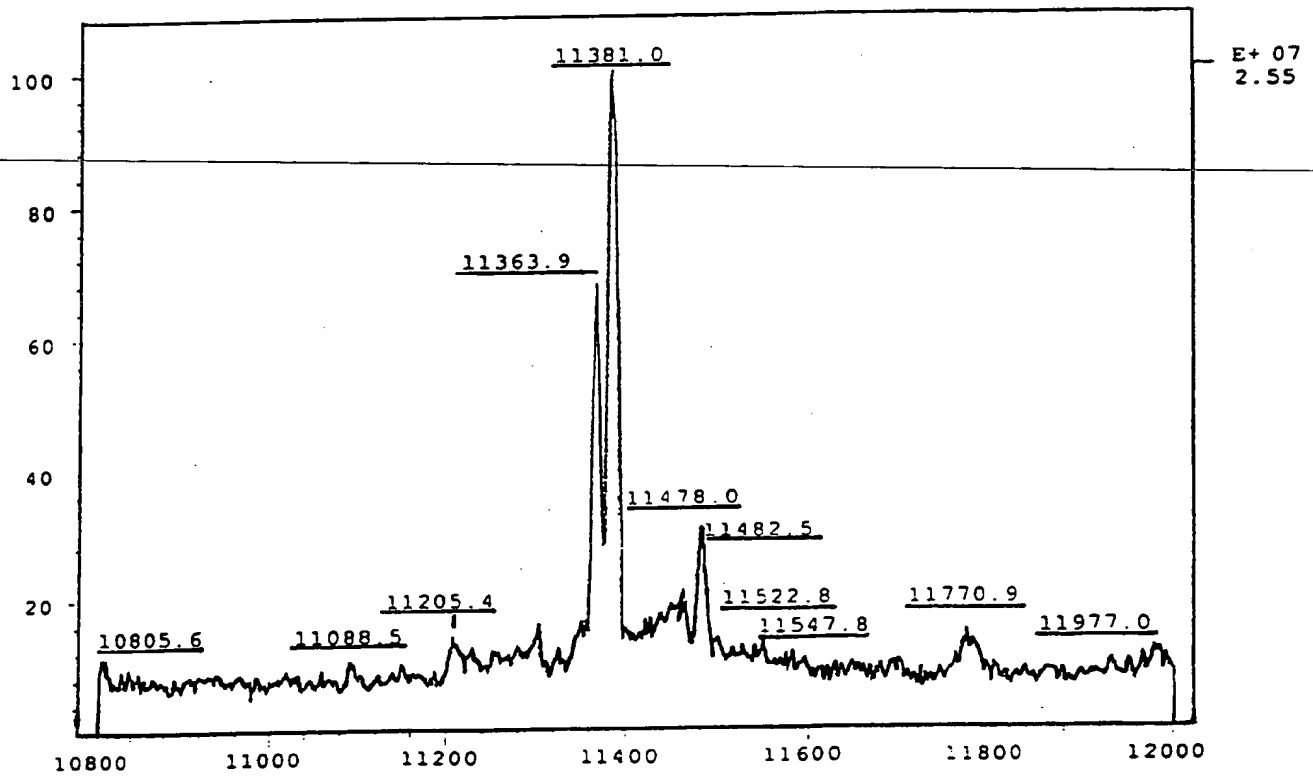
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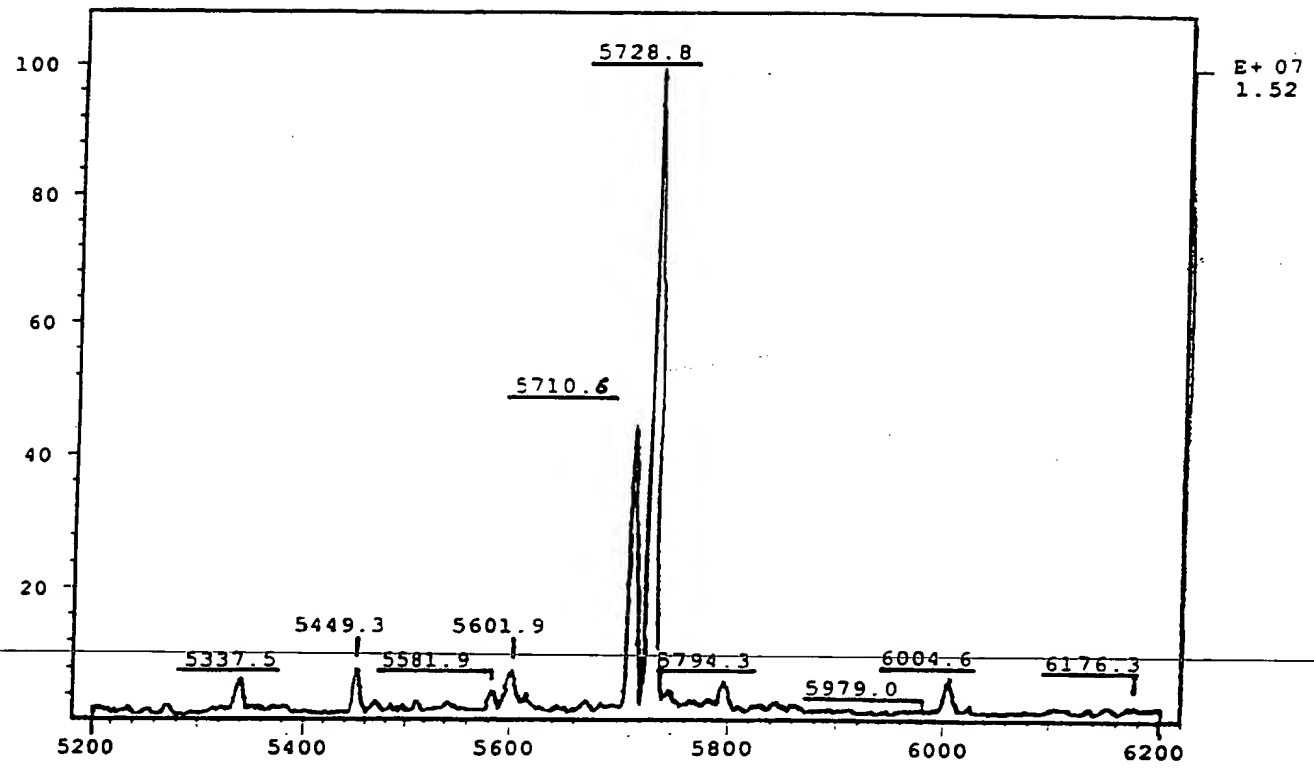
Figur 8

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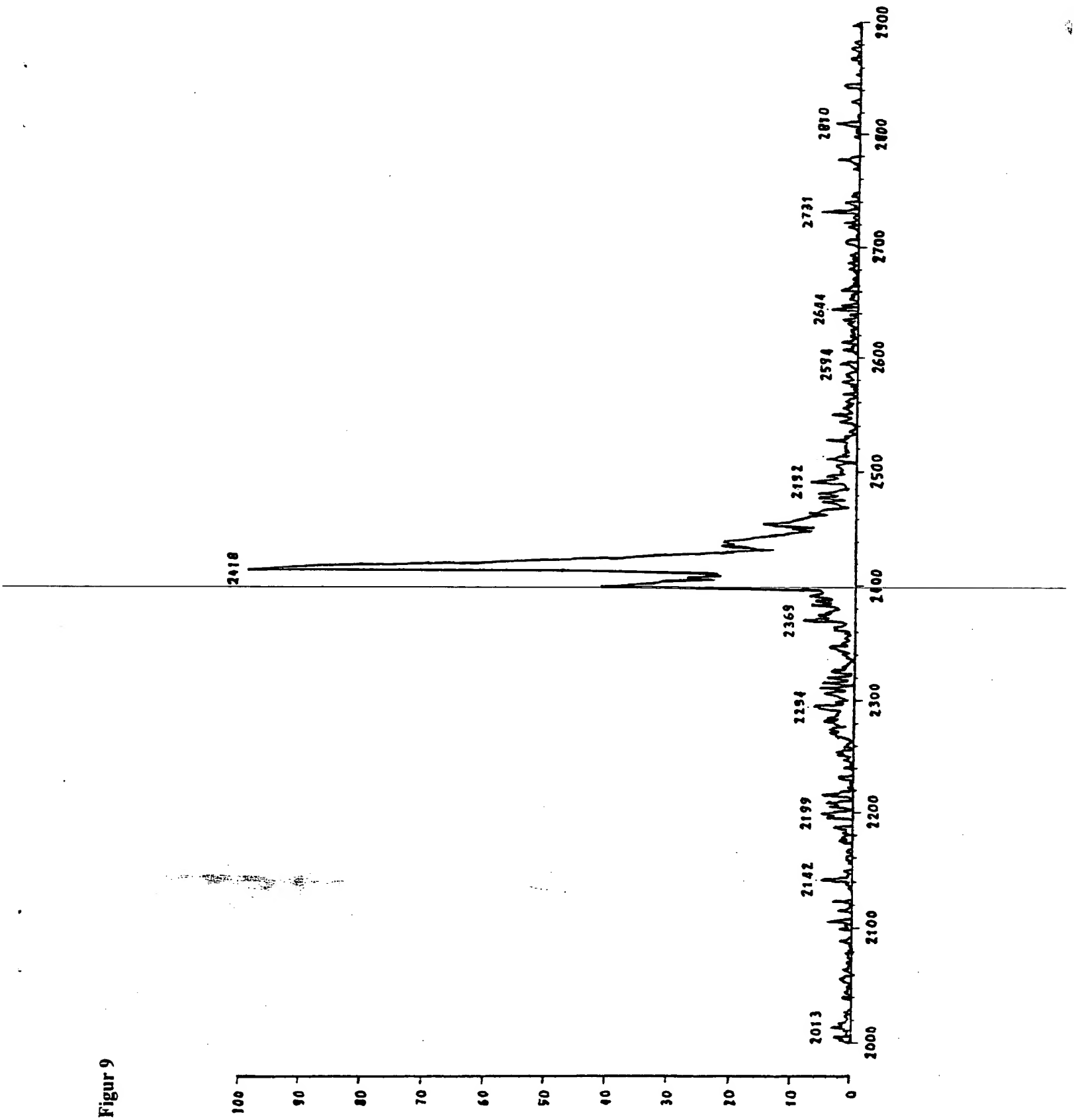
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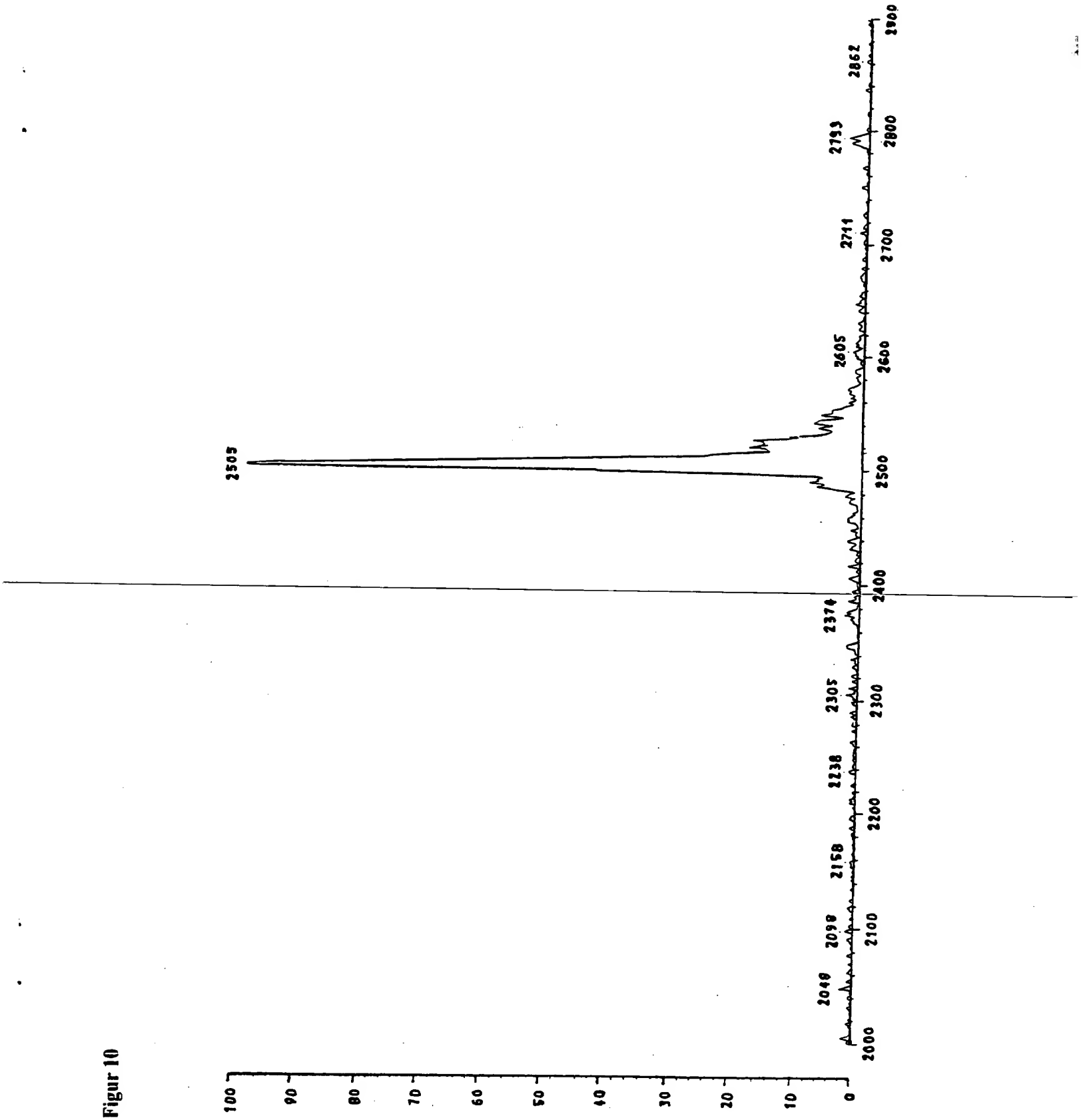
Figur 9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2300
2200
2100
2000

Figur 10

Not Cited

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 330 359 A2**

# (12) EUROPEAN PATENT APPLICATION

(21) Application number: 89301364.9

(22) Date of filing: 14.02.89

(51) Int. Cl.4: C07K 7/08 , A61K 39/21 , A61K 39/42 , C12P 21/00 , G01N 33/569 , C12N 15/00 , //C07K1/04

Claims for the following Contracting State: ES.

(30) Priority: 25.02.88 US 160378

(43) Date of publication of application: 30.08.89 Bulletin 89/35

(84) Designated Contracting States: CH DE ES FR GB IT LI

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## (54) Composition useful in the diagnosis and treating of HIV-1 infection.

(57) Methods and compositions for HIV-1 diagnosis, treatment, and vaccination include one or more peptides which correspond to antigenic determinants encoded by conserved regions of the HIV-1 genome. The peptides will be immunologically cross-reactive with such antigenic determinant sites and, preferably, will be capable of eliciting a serum-neutralizing response when administered to an individual who may or may not be infected with HIV-1. Antibodies to such peptides will also find use in the present invention.

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# COMPOSITIONS USEFUL IN THE DIAGNOSIS AND TREATING OF HIV-1 INFECTION

The present invention relates generally to th diagnosis and treatment of viral infections, and more particularly to the preparation of compositions useful for th diagnosis of and immunization against human immunodeficiency virus (HIV-1) infection.

Human immunodeficiency virus (HIV-1), previously designated HTLV-III and lymphoadenopathy-associated virus (LAV), has now been identified as the etiological agent responsible for acquired immunodeficiency syndrome (AIDS). As the spread of AIDS threatens to become the most deadly infectious disease of the twentieth century, methods for diagnosing and immunizing against HIV-1 infection become increasingly important.

A particular problem in both HIV-1 detection and immunization has been the considerable genetic variability of the virus. HIV-1 comprises numerous variants, differing by as few as 80 nucleotides and as many as 1000 or more nucleotides. As the HIV-1 genome comprises only 9500 base pairs, it would be expected that there will be considerable variations in the antigenic characteristics of the various strains. Such variations in the antigenic character of the various strains, in turn, render the task of preparing effective diagnostic and therapeutic compositions much more difficult.

One hopeful approach for the preparation of HIV-1 diagnostics and vaccines has been to identify conserved or non-variable regions among the genomes of the various strains. Such conserved regions which encode immunologically recognizable antigens on the viral core and envelope would ideally serve as the basis for preparing immunological reagents which are cross-reactive with a wide range or preferably all strains of HIV-1.

Unfortunately, while numerous peptides corresponding to conserved regions within the HIV-1 genome have been identified, none have yet provided entirely reliable diagnostic reagents. Moreover, none of the conserved peptides have been proven to be at all effective in preparing vaccines or other therapeutic compositions.

For these reasons, it would be desirable to identify additional conserved regions within the HIV-1 genome which encode viral antigens which are immunologically reactive on a large number, preferably all, of the HIV-1 strains. Alternatively, it would be desirable to identify groups of conserved or non-conserved peptides which, when the individual peptides of the group are employed in combination, can be used as reagents in detecting the presence of HIV-1 antibodies to HIV-1 in patient samples. It would be particularly desirable if such conserved peptides or groups of peptides could be effectively employed in vaccines, typically referred to as subunit vaccines, where they could be used to elicit serum-neutralizing antibodies capable of reacting with a large number of HIV-1 strains when administered to individuals as a vaccine.

Preparation of HIV-1 peptides is described in several references. A 21 amino acid peptide corresponding to a sequence encoded by gp41 is described in European Patent Application No. 86/303224.9, Wang et al. (1987) ACPR 21:34, and Proc. Natl. Acad. Sci. USA 83:6159-6163. Kennedy et al. (1986) Science 231:1556-1559 describe an 18 amino acid peptide corresponding to a sequence encoded by gp160, the precursor to gp20 and gp41. Rosen et al. (1987) in: "Clinical Laboratory Molecular Analysis:Future Directions" pp. 229-236 and Smith et al. (1987) J. Clin. Microbiol. 25:1498-1504 describe the preparation of various peptides encoded by various regions on the gag and env genes. Wong-Staal et al. (1986) in U.S. Patent Application Serial No. 779,431 (available from NTIS) describe 14 and 17 amino acid peptides which are reactive with HIV-1. U.S. Patent No. 4,629,783 describes a number of peptides corresponding to sequences on gp110 and gp25 which are reactive with HIV-1. Shoeman et al. (1987) Anal. Biochem. 161:370-379 describe several peptides encoded by portions of the gag and env genes which are reactive with HIV-1.

Peptides corresponding substantially to epitopic or antigenic determinant sites encoded by the HIV-1 genome are useful in formulating diagnostic, therapeutic, and vaccine compositions. The peptides may be highly conserved within the various strains of HIV-1, or may be less conserved peptides where it is desired to utilize cross-reactivity with a limited number (i.e., fewer than all) of HIV-1 strains. The less conserved peptides may also find use in combination with other more or less conserved peptides where the combination of peptides is cross-reactive with most or all HIV-1 strains. Preferably, the peptides or groups of peptides will be able to elicit a serum neutralizing immune response when administered to a normal or HIV-1-infected individual. Additionally, antibodies prepared against th peptides or groups of peptides will be useful in formulating diagnostic and therapeutic compositions and may be used to affinity purify monospecific antibodies from polyclonal sera.

In a preferred aspect of the present inv ntion, the peptides ar synthetically-produced linear fragments having a length in the range from about 6 to 50 amino acids, usually being in the range from about 8 to 25

amino acids. Use f such synthetic fragments is advantageous in that large quantities of the peptides may be produced without purification from an HIV-1 source. Moreover, the synthetic peptides are free from bacterial and yeast components which are normally present in recombinantly-produced peptides. Surprisingly, a plurality of linear fragments have been identified which are cross-reactive with high percentages of HIV-1 strains derived from diverse geographical locations. These linear fragments may be employed in combination to identify HIV-1-infected sera employing conventional immunoassays, particularly ELISA'S and dot blots. Usually, at least two immunologically distinct peptides will be employed in the assay procedure, more usually at least four, and frequently eight or more. Such assays may be employed as well for pathogens other than HIV-1, including both viral and bacterial pathogens.

In a second preferred aspect of the present invention, conserved peptides encoded by the regulatory regions of the HIV-1 genome have been prepared and used to screen for HIV-1-infected sera. Detection of antibodies to the regulatory proteins in sera appears to be highly diagnostic of HIV-1 infection and may show a correlation with disease state. Particular peptides encoded by the tat, 3′orf, Rorf, Uorf, trs/art and sor regions of the HIV-1 genome have been identified.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

According to the present invention, novel compositions and methods are provided for detecting, inhibiting, and neutralizing HIV-1 infection. The compositions include peptides having an amino acid sequence which is substantially the same as that encoded by various conserved and non-conserved regions of the HIV-1 genome. The compositions also comprise groups of peptides corresponding to immunologically distinct regions on the HIV-1 regulatory proteins. The individual peptides are selected so that the groups are immunologically cross-reactive with most and preferably all strains of HIV-1. The peptides and groups of peptides will correspond to immunologically-reactive sites on the HIV-1 particle, and preferably will mimic a neutralizing region on the virus. The compositions of the present invention also include antibodies raised against the peptides.

HIV-1 is classed as a retrovirus and includes a ribonucleoprotein core surrounded by a lipid-containing envelope. The HIV-1 genome is more complex than most other known retroviruses, and each end of the provirus includes long terminal repeats which regulate transcription. At least seven genes are encoded by the genome, including the gag, pol, and env genes which encode the core proteins, reverse transcriptase, and envelope proteins respectively. The core proteins include g18, while the envelope proteins include gp41 and gp120, which are derived from precursor gp160. The genes encoding the regulatory proteins include tat, orf, trs/art, and sor. The tat gene appears to regulate the transcription and possibly the translation of mRNA. The trs gene appears to affect the distribution of the various forms of mRNA, and promote structural protein synthesis. The roles of the orf and sor genes are not known.

The term "neutralizing region" refers to those portions of the HIV-1 genome which encode epitopes reactive with antibodies which are capable of neutralizing HIV-1 infection in a human host. Suitable assays for neutralization are well known and may be based on reduction of HIV-1 infection in T-cell lines, reduction of plaque forming units (pfu) of HIV-1 pseudotypes bearing the envelope or core glycoproteins of HIV-1, syncytial inhibition tests, and virion-receptor binding tests. Alternatively, the neutralizing activity can be compared to antibody reactivity in immunochemical tests, such as immunofluorescence, immunoblot, and radioimmunoprecipitation assays.

The peptides of interest correspond to certain amino acid sequences encoded by the env, gag, pol, tat, orf, trs/art, and sor regions of the HIV-1 genome. More specifically, the peptides are fragments of gp120 and gp41 encoded by the env gene, p18 and p24 encoded by the gag gene, p32 encoded by the pol gene, and the regulatory proteins encoded by the tat, orf, trs/art, and sor genes. The sequences and locations. within the genes for the particular peptides are set forth in Table 1.

TABLE 1

| Peptide Designation | Gene | Amino Protein | Amino Acid Acid Nos. | Sequence* |
|---|---|---|---|---|
| 3 | gag | p18 | 1-15 | MGARASVLSGGELDP |
| 4 | gag | p18 | 11-25 | GELORWEKIRLRPGG |
| 5 | gag | p18 | 21-35 | LRPGGKKKYKLKHIV |
| 7 | gag | p18 | 41-55 | LERFAVNPGLLETSE |
| 9 | gag | p18 | 61-75 | LGQLQPSLQTGSEEL |
| 10 | gag | p18 | 71-85 | GSEELRSLINTVATL |
| 11 | gag | p18 | 81-95 | TVATLYCVHQRIEIK |
| 12 | gag | p18 | 91-105 | RIEIKDTKEALDKIE |
| 23 | gag | p24 | 221-235 | GPIAPGQMREPRGSD |
| 24 | gag | p24 | 231-245 | PRGSDIAGTTSTLQE |
| 25 | gag | p24 | 241-255 | STLQEQIGWMTNNPP |
| 26 | gag | p24 | 251-265 | TNNPPIPVGEITKRW |
| 28 | gag | p24 | 261-275 | IYKRWIILGLNKIVR |
| 36 | gag | p24 | 351-365 | QGVGGPGHKARVLAE |
| 37 | gag | p24 | 361-375 | RVLAEAMSQVTNTAT |

| Peptide Designation | Gene | Amino Protein | Amino Acid Acid Nos. | Sequence* |
|---|---|---|---|---|
| 180 | gag | p15 | 398-412 | EGHTARNCRAPRKKG |
| 181 | gag | p15 | 408-422 | PRKKGCWKCGKEGHQ |
| 182 | gag | p15 | 418-432 | KEGHQMKDCTERQAN |
| 184 | gag | p15 | 438-452 | WPSYKGRPGNPLQSR |
| 189 | gag | p15 | 488-502 | QEPIDKELYPLTSLR |
| 40 | env | gp120 | 11-25 | WRWGWRWGTMLLGML |
| 42 | env | gp120 | 31-45 | TEKLWVTVYYGVPVW |
| 43 | env | gp120 | 41-55 | GVPVWKGATTTLFCA |
| 50 | env | gp120 | 110-125 | LWDQSLKPCVKLTPL |
| 51 | env | gp120 | 121-135 | KLTPLCVSLKCTDLK |
| 52 | env | gp120 | 131-145 | CTDLKNDTNTNSSSG |
| 55 | env | gp120 | 161-175 | ISTSIRGKVQKEYAF |
| 61 | env | gp120 | 231-245 | KTFNGTGPCTNVSTV |
| 62 | env | gp120 | 241-255 | NVSTVQCTHGIRPVV |
| 63 | env | gp120 | 251-265 | IRPVVSTQLLLNGSL |
| 68 | env | gp120 | 301-315 | NNTRKSIRIQRGPGR |
| 82 | env | gp120 | 441-455 | GQIRSCCNITGLLLT |
| 84 | env | gp120 | 461-475 | SNNESEIFRPGGGDM |
| 87 | env | gp120 | 491-505 | IEPLGVAPTKAKRRV |
| 88 | env | gp120 | 501-515 | AKRRVVQREKRAVGI |
| 89 | env | gp41 | 511-525 | RAVGIGALFLGFLGA |
| 90 | env | gp41 | 521-535 | GFLGAAGSTMGAASM |
| 91 | env | gp41 | 531-545 | GAASMTLTVQARQLL |
| 92 | env | gp41 | 541-555 | ARQLLSGIVQQQNNL |
| 93 | env | gp41 | 551-565 | QQNNLLRAIEAQQHL |
| 94 | env | gp41 | 561-575 | AQQHLLQLTVWGIKQ |
| 99 | env | gp41 | 591-605 | QLLGIWGCSGKCICT |
| 100 | env | gp41 | 601-615 | KLICTTAVPWNASWS |
| 101 | env | gp41 | 611-625 | NASWSNKSLEQIWNN |
| 102 | env | gp41 | 621-635 | QIWNNMTWMEWDREI |
| 103 | env | gp41 | 631-645 | WDREINNYTSLIHSL |

| Peptide Designation | Gene | Amino Protein | Amino Acid Acid Nos. | Sequence* |
|---|---|---|---|---|
| 105 | env | gp41 | 651-665 | NQQEKNEQELLELDK |
| 106 | env | gp41 | 661-675 | LELDKWASLWNWFNI |
| 107 | env | gp41 | 671-685 | NWFNITNWLWYIKLF |
| 108 | env | gp41 | 681-695 | YIKLFIMIVGGLVGL |
| 112 | env | gp41 | 721-735 | LPIPRGPDRPEGIEE |
| 114 | env | gp41 | 741-755 | DRDRSIRLVNGSLAL |
| 119 | env | gp41 | 791-805 | EALKYWWNLLQYWSQ |
| 120 | env | gp41 | 801-815 | QYWSQELKNSAVSLL |
| 122 | env | gp41 | 821-835 | AVAEGTDRVIEVVQG |
| 130 | pol | p32 | 625-639 | VTNKGRQKVVPLTNT |
| 131 | pol | p32 | 635-649 | PLTNTTNQKTELQAI |
| 132 | pol | p32 | 645-659 | ELQAIYLALQDSGLE |
| 139 | pol | p32 | 715-729 | VDKLVSAGIRKILFL |
| 143 | pol | p32 | 755-769 | LPPVVAKEIVASCDK |
| 144 | pol | p32 | 765-779 | ASCDKCQLKGEAMHG |
| 147 | pol | p32 | 795-809 | LEGKVILVAVHVASG |
| 155 | pol | p32 | 875-889 | QGVVESMNKELKKII |
| 156 | pol | p32 | 885-899 | LKKIIGQVRDQAEHL |
| 167 | pol | p32 | 995-1009 | IRDVGKQMAGDDCVA |
| 168 | pol | p32 | 1000-1015 | QMAGDDCVASRQDED |
| 169 | tat | p__ | 1-15 | MEPVDPRLEPWKHPG |
| 383 | u-orf | p27 | 1-15 | MQPIQIAIVALVVAI |
| 384 | u-orf | p27 | 11-25 | LVVAIIIAIVVWSIV |
| 388 | u-orf | p27 | 31-45 | KILRQRKIDRLIDRL |
| 389 | u-orf | p27 | 41-55 | LIDRLIERAEDSGNE |
| 393 | r-orf | p__ | 1-15 | MEQAPEDQGPQREPH |
| 394 | r-orf | p__ | 11-25 | QREPHNEWTLELLEE |
| 397 | r-orf | p__ | 41-55 | GLGQHIYETYGDTWA |

| Peptide Designation | Gene | Amino Protein | Amin Acid Acid Nos. | Sequence* |
|---|---|---|---|---|
| 403 | trs/art | p__ | 21-35 | FLYQSNPPPNPEGTR |
| 406 | trs/art | p__ | 51-65 | QIHSISERILGTYLG |
| 409 | trs/art | p__ | 81-95 | LTLDCNEDCGTSGTQ |
| 420 | sor | p23 | 71-85 | GLHTGERDWHLGQGV |
| 421 | sor | p23 | 81-95 | LGQGVSIEWRKKRYS |
| 422 | sor | p23 | 91-105 | KKRYSTQVDPELADQ |
| 423 | sor | p23 | 101-115 | ELADQLIHLYYFDCF |
| 427 | sor | p23 | 141-155 | KVGSLQYLALAALIT |
| 428 | sor | p23 | 151-165 | AALITPKKIKPPLPS |
| 429 | sor | p23 | 161-175 | PPLPSVTKLTEDRWN |

* Nucleotide and amino acid sequence numbering is based on the published sequence for the HBX2 and BH10 strains (Human Retrovirus and AIDS, Myers et al., eds., Los Alamos National Laboratory, Los Alamos, New Mexico 87545 (1987)).

The amino acid sequences of the peptides of the present invention need not correspond precisely to the sequences set forth in the above table. Rather, it is essential only that the peptides define at least one epitope or antigenic determinant which is immunologically cross-reactive with the enumerated sequences. Thus, peptides of the present invention may include only a portion of the listed amino acid sequences, but must include at least 6 contiguous amino acids, usually including at least 9 amino acids, and typically including at least 12 or more amino acids of any one of the sequences.

The peptides of the present invention may also include additional amino acid sequences not corresponding to those enumerated in the above Table. Such additional sequences may be present at either the C-terminus, N-terminus, or both and can either be synthesized at the same time as the listed sequences, or may be conjugated or attached to the sequences by well known techniques. Usually, such additional sequences may be useful in separation, conjugation, or other manipulation of the peptides which facilitate their use in some known manner. In particular, the peptides may include additional amino acids or be otherwise modified at the C-terminus or N-terminus to provide for binding or conjugation of the peptide to a solid phase or another protein. For example, a gly-gly-cys sequence may be added to either terminus to facilitate coupling to a carrier. Hydrophobic residues or lipid-containing moieties may be added to enhance liposome or membrane binding. While there is no theoretical upper limit to the size of the peptides of the present invention, there will seldom be reason to prepare peptides having a length greater than 100 amino acids, and the peptides will usually be shorter than 75 amino acids.

The peptides of the present invention may also embody substitutions of particular amino acids, although there will usually be no substitutions in shorter peptides of only six amino acids. Normally, there will be no more than one substitution in sequences from 6 to about 10 amino acids, and no more than two substitutions in sequences having a length from about 10 to 20 amino acids. Substitutions, of course, must not substantially alter the immunological characteristic of the peptide in an adverse manner and may result in enhanced binding or antigenic properties. The cross-reactivity with the particular epitope or antigenic determinant site must not be substantially reduced.

Depending on its length and location, th peptide of the present invention may be substantially free from folding, may display folding which is not characteristic of the natural gene product, or may display a natural or substantially natural folding. Usually, the shorter fragments of 25 amino acids and below will

display minimal or non-natural folding, while the longer fragments ar more likely to display folding which is similar to the natural product.

The peptides of the present invention may also be incorporated in compositions for the prophylactic and/or therapeutic treatment of HIV-1 infection. In therapeutic applications, the compositions are administered to a patient already infected with HIV-1, in an amount sufficient to cure or at least partially inhibit the infection and its complications. An amount adequate to accomplish this is defined as a therapeutically effective dose. Precise determination of such a dose will depend on the severity of the infection and the general state of the patient's own immune system, but will generally range from about 1 to 200 milligrams of peptide per kilogram of body weight, typically being in the range from about 5 to 25 milligrams per kilogram of body weight.

In prophylactic applications, the peptides of the present invention are administered to an individual who is not infected with HIV-1, but who will usually be at risk of being exposed to the virus. In such prophylactic applications, the peptides will be formulated in a vaccine composition intended to elicit a protective immune response. The vaccine compositions will include a prophylactically effective dose, which will again depend on the patient's state of health and general level of immunity. Usually, the dosage will be somewhat less than for therapeutic applications, typically being in the range from about 0.1 milligrams to 25 milligrams per kilogram of body weight, usually being in the range from about 0.5 milligrams to 2.5 milligrams per kilogram of body weight.

Single of multiple administrations of the peptide compositions may be carried out with the exact dose level and pattern of administration being selected by the treating physician.

The peptides will be incorporated in a physiologically-acceptable carrier, and will be injected intravenously, subcutaneously, intramuscularly, intraperitoneally, or the like. Suitable carriers include phosphate buffered saline, saline, water, potassium chloride, sodium lactate, and the like. The concentration of the peptide in the carrier will vary depending on the ultimate use, activity, and mode of administration.

The peptides of the present invention may be natural, i.e., fragments of protein isolated from HIV-1, but will more usually be synthetic. The natural proteins may be isolated from HIV-1 by conventional techniques such as high performance liquid chromatography (HPLC) and affinity chromatography, but care should be taken that no intact HIV-1 or infectious materials are left in the isolated compositions. Conveniently, polyclonal or monoclonal antibodies obtained according to the present invention (as described below) may be used to prepare a suitable affinity column by well known techniques. Such techniques are taught, for example, in Hudson and Hay, Practical Immunology, Blackwell Scientific Publications, Oxford, United Kingdom, 1980, Chapter 8. The peptides may then be obtained by chemical or enzymatic cleavage of the intact protein.

Because of the difficulty and danger in isolating the peptides of the present invention from HIV-1, however, it is preferred to produce synthetic fragments based on the HIV-1 amino acid sequences as set forth above. Synthetic polypeptides which are immunologically cross-reactive with the natural HIV-1 proteins may be produced by either of two general approaches. First, polypeptides having fewer than about 100 amino acids, usually fewer than about 50 amino acids, and more usually 25 or fewer amino acids, may be synthesized by the well-known Merrifield solid-phase chemical synthesis method where amino acids are sequentially added to a growing chain (Merrifield (1963) J. Am. Chem. Soc. 85:2149-2156). Specific synthesis techniques are described below.

Linear peptides may be chemically synthesized by manual means or automatically in commercially-available synthesis equipment. Systems for manually synthesizing peptides on polyethylene pegs are available from Cambridge Research Biochemicals. An exemplary manual technique for preparing peptides is described in detail in the Experimental section hereinafter. Automatic peptide synthesis equipment is available from suppliers including Applied Biosystems, Inc., Foster City, California, Beckman Instruments, Inc., Waldwick, New Jersey, and Biosearch, Inc., San Rafael, California. Using such automatic synthesizers according to manufacturer's instructions, peptides may be produced in gram quantities for use in the present invention.

The use of relatively short linear peptide fragments has a number of advantages in performing the methods of the present invention. The peptides may be produced in quantity and free from contaminating substances which are found in recombinantly produced peptides. Moreover, the risks associated with obtaining peptides from HIV-1 itself are entirely avoided. Short, linear peptides are particularly effective when employed in groups of at least two, frequently four, and often eight or more. By properly selecting the peptides from different conserved and non-conserved regions of the HIV-1 genome, assays can be devised which are capable of detecting infection with most and preferably all HIV-1 strains. Specific assay protocols utilizing groups of linear peptide fragments are described in more detail hereinafter.

The second general approach for synthesizing the peptides of the present invention involves the

expression in cultured cells of recombinant DNA molecules encoding a desired portion of the HIV-1 env, gag or pol gene. The HIV-1 gene may itself be natural or synthetic. Conveniently, polynucleotides may be synthesized by well-known techniques. For example, short single-stranded DNA fragments may be prepared by the phosphoramidite method described by Beaucage and Carruthers (1981) Tett. Letters 22:1859-1862. A double-stranded fragm nt may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

The natural or synthetic DNA fragments coding for a desired HIV-1 fragment will be incorporated in DNA constructs capable of introduction to and expression in an in vitro cell culture. Usually, the DNA constructs will be suitable for replication in a unicellular host, such as yeast or bacteria, but may also be intended for introduction and integration within the genome of cultured mammalian or other eukaryotic cell lines. DNA constructs prepared for introduction into bacteria or yeast will include a replication system recognized by the host, the HIV-1 DNA fragment encoding the desired polypeptide product, transcriptional and translational initiation regulatory sequences joined to the 5′-end of the HIV-1 DNA sequence, and transcriptional and translational termination regulatory sequences joined to the 3′-end of the HIV-1 sequence. The transcriptional regulatory sequences will include a heterologous promoter which is recognized by the host. Conveniently, available expression vectors which include the replication system and transcriptional and translational regulatory sequences together with an insertion site for the HIV-1 DNA sequence may be employed.

To be useful in the detection and immunization methods of the present invention, the peptides are obtained in substantially pure form, that is, typically about 50% w/w or more purity, substantially free of interfering proteins and contaminants. Preferably, the HIV-1 peptides are isolated or synthesized in a purity of at least about 80% w/w and, more preferably, in at least about 95% w/w purity. Using conventional purification techniques, homogeneous peptides of at least 99% w/w can be obtained. For example, the peptides may be purified by use of reverse-phase high performance liquid chromatography. Usually, however, it is not essential to obtain highly pure peptide, particularly when using the peptides as a binding reagent in diagnostic assays.

Antibodies to the HIV-1 peptides may be obtained by injecting the purified peptide into a wide variety of vertebrates in accordance with conventional techniques. Suitable vertebrates include mice, rats, sheep, and goats, and in particular mice. Usually, the animals are bled periodically with successive bleeds having improved titer and specificity. The antigens may be injected intramuscularly, intraperitoneally, subcutaneously, or the like. Usually, a vehicle is employed, such as a complete or incomplete Freund's adjuvant. If desired, monoclonal antibodies can be prepared. Alternatively, the peptides may be used to isolate monospecific antibodies from a natural source, i.e., from the sera of an individual infected with HIV-1.

To obtain monoclonal antibodies, spleen cells from the immunized vertebrate are immortalized. The manner of immortalization is not critical. Presently, the most common method is fusion with a myeloma fusion partner. Other techniques include EBV transformation, transformation with bare DNA, e.g., oncogenes, retroviruses, etc., or any other method which provides for stable maintenance of the cell line and production of monoclonal antibodies. Human monoclonal antibodies may be obtained by fusion of the spleen cells with an appropriate human fusion partner. A detailed technique for producing mouse x mouse monoclonal antibodies is taught by Oi and Herzenberg, in "Selected Methods in Cellular Immunology," Mishell and Shiigi (eds.), W. H. Freeman and Co., San Francisco (1980), pp. 351-372. The antibodies of the present invention may be of any immunoglobulin class, i.e, IgG, including IgG1, IgG2A, and IgG2B, IgA, IgD, IgE, and IgM, usually being IgG or IgM.

Once antibodies having suitable specificity have been prepared, a wide variety of immunological assay methods are available for detecting the HIV-1 in a biological sample. Numerous competitive and non-competitive protein binding assays have been described in the scientific and patent literature, and a large number of such assays are commercially available.

In performing immunoassays according to the present invention, it will usually be necessary to pretreat the biological sample in some manner. Sample preparation will vary depending on the source of the biological sample. Serum samples will typically be prepared by clotting whole blood and isolating the supernatants in accordance with well known methods. Other biological fluids, such as semen, sputem, and urine, may also be prepared by conventional techniques. Solid tumors and other tissue samples will usually be prepared by lysing the cells and solubilizing the cellular components of interest.

The peptides of the present invention are particularly useful as reagents in solid phas binding assays where antibodies in a patient sample are immobilized by peptides bound to a solid phase. While patient samples may be screened against a single peptide, it will normally be preferred to utilize a group or panel of peptides where the individual peptides in the group are selected to maximize the probability of binding

HIV-1 antibodies. Because of the heterogeneity of the HIV-1 strains and variations in an individual's immune response (i. ., certain individual patients may be incapable of producing a d tectabl level of antibodies to particular epitopes on the viral particle), detection based on antibody reactivity with a single peptide will generally be inadequate.

The individual peptides in such groups will also be selected to avoid cross-reactivity with viruses other than HIV-1, such as HIV-2. The avoidance of such cross-reactivity is critical in avoiding false positive test results.

The individual peptides in such groups may be employed simultaneously or discretely. Normally, the peptides will be mixed and attached to a single solid phase and the assays then run in a conventional manner (exemplary protocols are then set forth below). Binding of antibody in the patient sample to one or more of the peptides will result in a positive test, while failure of all peptides to bind antibody is a highly reliable indication that the patient is free from HIV-1 antibodies. In some cases, however, it may be desirable to separately test for the reactivity of each individual peptide, or a subgrouping of peptides. The reactivity profiles obtained by such individual screening will provide more detailed information on the patient's immune response which may be of substantial value.

The present invention also provides for the detection of HIV-1 regulatory proteins in patient samples, particularly serum samples. The regulatory proteins may be detected simultaneously with or separately from the structural peptides. Because of the relatively low concentration of the regulatory proteins in the patient serum, it will usually be desirable to utilize high regulatory peptide concentrations on the solid phase to assure detection of antibodies in the patient sample.

The peptides and antibodies of the present invention are useful in virtually any type of immunological detection, including homogeneous and non-homogeneous (solid phase) immunoassays, competitive and non-competitive assays, and immunohistochemical staining techniques. Preferred are dot blot and enzyme-linked immunosorbent assays (ELISA), and specific protocols for such assays are set forth hereinbelow.

The following examples are offered by way of illustration, not by way of limitation.

## EXPERIMENTAL

Two hundred thirty five peptides 15 amino acids in length were synthesized according to the simultaneous multiple peptide synthesis method of Geysen et al. (1985) Proc. Natl. Acad. Sci. USA 81:3998 and 82:178, on polyethylene rods functionalized with a six-carbon aliphatic diamine spacer coupled to FMOC-$\beta$-alanine. The rods were obtained from Cambridge Research Biochemicals, Cambridge, United Kingdom. FMOC-protected amino acids active esters were added to the spacer, where the active esters were pentafluorophenyl esters except for serine and threonine which were 1-oxo-2-dihydroxyldihydrobenzotriazine esters. Following the synthesis, the peptide side-chain protecting groups were removed, leaving the peptides still attached to the supports. The sequences of the peptides were based on the predicted sequence of HIV-1 strains HBX2 and BH10 and selected to cover the entire amino acid sequence of all HIV-1 gene products. Consecutive peptides overlapped by five amino acids, and each peptide was tested for reactivity with a panel of HIV-1 positive and negative sera. The test was performed according to the ELISA protocol set forth hereinafter. Of the 235 peptides tested, 84 were found to react with at least 60% of the HIV-1 positive samples and with none of the HIV-1 negative samples. These 84 peptides are listed in Table 1 hereinabove.

Selected ones of the 84 peptides were then tested as follows. PEG pins containing HIV-1 synthetic peptide 15-mers were preincubated with Blotto solution for 30 minutes on a shaker at room temperature (RT). Pins were then washed twice with PBS-Tween®. These pins were immersed into the wells of a 96-well microtiter plate containing in each well 175 µl of HIV-1-positive or negative sera diluted to 1/100. After 1 hour of incubation at room temperature on a shaker, pins were initially washed with PBS-Tween® supplemented with 0.3 M NaCl in order to reduce the non-specific binding of human Ig onto pins. This was followed by two washes with PBS-Tween®. Then, the pins were again immersed into the wells of a 96-well microtiter plate containing 175 µl of anti-human Ig-alkaline phosphatase (Bio-Rad) for 30 minutes at RT on a shaker. Again, the pins were washed with PBS-Tween® supplemented with 0.3 M NaCl once, followed by two washes with PBS-Tween®. The substrate (p-nitro phenyl phosphate) dissolved in diethanolamin buff r (Bio-Rad) was added into the wells of 96-well microtiter plate (150 µl) and pins were immersed into these wells for 30 minutes at 37° C. The absorbance at 405 nm was recorded using a Titertek Plate Reader connected to the Microvax computer. Positivity was determined from the absorbance values above the background read from the bar graphs. Individual HIV-1 positive and negative sera were used except for HLR

P I, II and III, which each was a pool of ten individual positive sera obtained from Hoffman-La Roche.

Since the peptide-containing polyethylene rods were re-used in the screening of several antisera, they wer cleaned of any adhering antibodies or pr teins by heating at 60° C disrupti n buffer for 30 minutes. This buff r consisted of 1% sodium dodecyl sulfate and 0.1% 2-m rcaptoethanol in sodium phosphate buffer, pH 7.2. Following a final wash in boiling methanol, the rods were ready for re-use.

In order to test each more thoroughly, the 84 peptides of Tabl 1 were synthesized in greater quantity by conventional automated and manual solid-phase techniques. The peptides were evaluated for their utility as binding reagents in both ELISA and dot blot assays.

The peptides were used in dot blot membrane assays for the detection of HIV-1 specific antibodies in human sera as follows. The peptides were dissolved in phosphate-buffered saline (PBS) containing 0.1% sodium azide at a concentration of 2 mg/ml. Approximately 1 microliter of this solution was spotted directly onto a nitrocellulose membrane (Gelman), and the membranes dried at 45° for 2 hours. The membrane was washed twice for 20 minutes each with PBS containing 0.3% Tween®-20 and then distilled water.

A 1% dilution of individual sera in wash diluent (0.5% non-fat dry milk powder In PBS containing 0.3% Tween®-20) was incubated over the peptide-spotted membranes for 60 minutes at room temperature. Unbound antibodies were removed by two 10-minute washes with wash diluent. Subsequently, goat anti-human IgG alkaline phosphatase conjugate in wash diluent was allowed to react for 30 minutes. Following two 10-minute washes with wash diluent, the membranes were incubated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) for 10 minutes. Positive sera were detected by the development of a purple color at the position of the peptide on the membrane. Negative sera showed no color change.

ELISA assays were performed as follows. Microtiter plates (96 well, Immulon-II) were coated with one or more peptides (250 ng - 1000 ng in 100 µl of PBS per well) by incubation at room temperature for 16-20 hours. The wells were then washed three times with PBS containing 0.3% Tween®-20. Serum samples were diluted 1:100 µl with wash diluent and 100 µl of sample was added to each well. After incubation at room temperature for 1 hour, the wells were washed six times with PBS containing 0.3% Tween®-20. A 100 µl aliquot of a 1:3000 dilution of goat anti-human IgG alkaline phosphatase in wash diluent was added to each well and then incubated at room temperature for 30 minutes. Following six washes with PBS containing 0.3% Tween®-20, p-nitrophenylphosphate substrate (100µl of a 1 mg/ml solution in diethanolamine buffer) was added to each well. The color was allowed to develop for 30 minutes and then read at 444nm in an ELISA plate reader.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

## Claims

1. Peptides having fewer than 100 amino acids and comprising at least one epitope defined by one of the following amino acid sequences:
MGARASVLSGGELDP; GELORWEKIRLRPGG; LRPGGKKKYKLKHIV;
LERFAVNPGLLETSE; LGQLQPSLQTGSEEL; GSEELRSLTNTVATL;
TVATLYCVHQRIEIK; RIEIKDTKEALDKIE; GPIAPGQMREPRGSD;
PRGSDIAGTTSTLQE; STLQEQIGWMTNNPP; TNNPPIPVGEITKRW;
IYKRWIILGLNKIVR; QGVGGPGHKARVLAE; RVLAEAMSQVTNTAT;
EGHTARNCRAPRKKG; PRKKGCWKCGKEGHQ; KEGHQMKDCTERQAN;
WPSYKGRPGNPLQSR; QEPIDKELYPLTSLR; WRWGWRWGTMLLGML;
TEKLWVTVYYGVPVW; GVPVWKGATTTLFCA; LWDQSLKPCVKLTPL;
KLTPLCVSLKCTDLK; CTDLKNDTNTNSSSG; ISTSIRGKVQKEYAF;
KTFNGTGPCTNVSTV; NVSTVQCTHGIRPVV; IRPVVSTQLLLNGSL;
NNTRKSIRIQRGPGR; GQIRSCCNITGLLLT; SNNESEIFRPGGGDM;
IEPLGVAPTKAKRRV; AKRRVVQREKRAVGI; RAVGIGALFLGFLGA;
GFLGAAGSTMGAASM; GAASMTLTVQARQLL; ARQLLSGIVQQQNNL;
QQNNLLRAIEAQQHL; AQQHLLQLTVWGIKQ; QLLGIWGCSGKCICT;
KLICTTAVPWNASWS; NASWSNKSLEQIWNN; QIWNNMTWMEWDREI;
WDREINNYTSLIHSL; NQQEKNEQELLELDK; LELDKWASLWNWFNI;
NWFNITNWLWYIKLF; YIKLFIMIVGGLVGL; LPIPRGPDRPEGIEE;
DRDRSIRLVNGSLAL; EALKYWWNLLQYWSQ; QYWSQELKNSAVSLL;

AVAEGTDRVIEVVQG; VTNKGRQKVVPLTNT; PLTNTTNQKTELQAI; ELQAIYLALQDSGLE; VDKLVSAGIRKILFL; LPPVVAKEIVASCDK; ASCDKCQLKGEAMHG; LEGKVILVAVHVASG; QGVVESMNKELKKII; LKKIIGQVRDQAEHL; IRDVGKQMAGDDCVA; QMAGDDCVASRQDED; MEPVDPRLEPWKHPG; MQPIQIAIVALVVAI; LVVAIIIAIVVWSIV; KILRQRKIDRLIDRL; LIDRLIERAEDSGNE; MEQAPEDQGPQREPH; QREPHNEWTLELLEE; GLGQHIYETYGDTWA; FLYQSNPPPNPEGTR; QIHSISERILGTYLG; LTLDCNEDCGTSGTQ; GLHTGERDWHLGQGV; LGQGVSIEWRKKRYS; KKRYSTQVDPELADQ; ELADQLIHLYYFDCF; KVGSLQYLALAALIT; AALITPKKIKPPLPS; and PPLPSVTKLTEDRWN.

2. Peptides as in claim 1, which are substantially free from natural folding.

3. Synthetic peptides as in claim 1, which are substantially free from glycosylation.

4. A synthetic peptide including a sequence substantially the same as one selected from the group consisting of:

MGARASVLSGGELDP; GELQRWEKIRLRPGG; LRPGGKKKYKLKHIV; LERFAVNPGLLETSE; LGQLQPSLQTGSEEL; GSEELRSLTNTVATL; TVATLYCVHQRIEIK; RIEIKDTKEALDKIE; GPIAPGQMREPRGSD; PRGSDIAGTTSTLQE; STLQEQIGWMTNNPP; TNNPPIPVGEITKRW; IYKRWIILGLNKIVR; QGVGGPGHKARVLAE; RVLAEAMSQVTNTAT; EGHTARNCRAPRKKG; PRKKGCWKCGKEGHQ; KEGHQMKDCTERQAN; WPSYKGRPGNPLQSR; QEPIDKELYPLTSLR; WRWGWRWGTMLLGML; TEKLWVTVYYGVPVW; GVPVWKGATTTLFCA; LWDQSLKPCVKLTPL; KLTPLCVSLKCTDLK; CTDLKNDTNTNSSSG; ISTSIRGKVQKEYAF; KTFNGTGPCTNVSTV; NVSTVQCTHGIRPVV; IRPVVSTQLLLNGSL; NNTRKSIRIQRGPGR; GQIRSCCNITGLLLT; SNNESEIFRPGGGDM; IEPLGVAPTKAKRRV; AKRRVVQREKRAVGI; RAVGIGALFLGFLGA; GFLGAAGSTMGAASM; GAASMTLTVQARQLL; ARQLLSGIVQQQNNL; QQNNLLRAIEAQQHL; AQQHLLQLTVWGIKQ; QLLGIWGCSGKCICT; KLICTTAVPWNASWS; NASWSNKSLEQIWNN; QIWNNMTWMEWDREI; WDREINNYTSLIHSL; NQQEKNEQELLELDK; LELDKWASLWNWFNI; NWFNITNWLWYIKLF; YIKLFIMIVGGLVGL; LPIPRGPDRPEGIEE; DRDRSIRLVNGSLAL; EALKYWWNLLQYWSQ; QYWSQELKNSAVSLL; AVAEGTDRVIEVVQG; VTNKGRQKVVPLTNT; PLTNTTNQKTELQAI; ELQAIYLALQDSGLE; VDKLVSAGIRKILFL; LPPVVAKEIVASCDK; ASCDKCQLKGEAMHG; LEGKVILVAVHVASG; QGVVESMNKELKKII; LKKIIGQVRDQAEHL; IRDVGKQMAGDDCVA; QMAGDDCVASRQDED; MEPVDPRLEPWKHPG; MQPIQIAIVALVVAI; LVVAIIIAIVVWSIV; KILRQRKIDRLIDRL; LIDRLIERAEDSGNE; MEQAPEDQGPQREPH; QREPHNEWTLELLEE; GLGQHIYETYGDTWA; FLYQSNPPPNPEGTR; QIHSISERILGTYLG; LTLDCNEDCGTSGTQ; GLHTGERDWHLGQGV; LGQGVSIEWRKKRYS; KKRYSTQVDPELADQ; ELADQLIHLYYFDCF; KVGSLQYLALAALIT; AALITPKKIKPPLPS; and PPLPSVTKLTEDRWN.

5. Synthetic peptides as in claim 1, which are substantially free from natural folding.

6. A synthetic peptide as in claim 4, which is substantially free from glycosylation.

7. Antibody reactive with at least one epitope defined by the following amino acid sequences:

MGARASVLSGGELDP; GELQRWEKIRLRPGG; LRPGGKKKYKLKHIV; LERFAVNPGLLETSE; LGQLQPSLQTGSEEL; GSEELRSLTNTVATL; TVATLYCVHQRIEIK; RIEIKDTKEALDKIE; GPIAPGQMREPRGSD; PRGSDIAGTTSTLQE; STLQEQIGWMTNNPP; TNNPPIPVGEITKRW; IYKRWIILGLNKIVR; QGVGGPGHKARVLAE; RVLAEAMSQVTNTAT; EGHTARNCRAPRKKG; PRKKGCWKCGKEGHQ; KEGHQMKDCTERQAN; WPSYKGRPGNPLQSR; QEPIDKELYPLTSLR; WRWGWRWGTMLLGML; TEKLWVTVYYGVPVW; GVPVWKGATTTLFCA; LWDQSLKPCVKLTPL; KLTPLCVSLKCTDLK; CTDLKNDTNTNSSSG; ISTSIRGKVQKEYAF; KTFNGTGPCTNVSTV; NVSTVQCTHGIRPVV; IRPVVSTQLLLNGSL; NNTRKSIRIQRGPGR; GQIRSCCNITGLLLT; SNNESEIFRPGGGDM; IEPLGVAPTKAKRRV; AKRRVVQREKRAVGI; RAVGIGALFLGFLGA;

GFLGAAGSTMGAASM; GAASMTLTVQARQLL; ARQLLSGIVQQQNNL;
QQNNLLRAIEAQQHL; AQQHLLQLTVWGIKQ; QLLGIWGCSGKCICT;
KLICTTAVPWNASWS; NASWSNKSLEQIWNN; QIWNNMTWMEWDREI;
WDREINNYTSLIHSL; NQQEKNEQELLELDK; LELDKWASLWNWFNI;
NWFNITNWLWYIKLF; YIKLFIMIVGGLVGL; LPIPRGPDRPEGIEE;
DRDRSIRLVNGSLAL; EALKYWWNLLQYWSQ; QYWSQELKNSAVSLL;
AVAEGTDRVIEVVQG; VTNKGRQKVVPLTNT; PLTNTTNQKTELQAI;
ELQAIYLALQDSGLE; VDKLVSAGIRKILFL; LPPVVAKEIVASCDK;
ASCDKCQLKGEAMHG; LEGKVILVAVHVASG; QGVVESMNKELKKII;
LKKIIGQVRDQAEHL; IRDVGKQMAGDDCVA; QMAGDDCVASRQDED;
MEPVDPRLEPWKHPG; MQPIQIAIVALVVAI; LVVAIIIAIVVWSIV;
KILRQRKIDRLIDRL; LIDRLIERAEDSGNE; MEQAPEDQGPQREPH;
QREPHNEWTLELLEE; GLGQHIYETYGDTWA; FLYQSNPPPNPEGTR;
QIHSISERILGTYLG; LTLDCNEDCGTSGTQ; GLHTGERDWHLGQGV;
LGQGVSIEWRKKRYS; KKRYSTQVDPELADQ; ELADQLIHLYYFDCF;
KVGSLQYLALAALIT; AALITPKKIKPPLPS; and PPLPSVTKLTEDRWN.

8. Monoclonal antibody as in claim 7.

9. Polyclonal antibody as in claim 7.

10. A composition used for eliciting an immune response against HIV-1 infection, said composition comprising a dose of a peptide including a sequence substantially the same as one selected from the group consisting of: MGARASVLSGGELDP;
GELQRWEKIRLRPGG; LRPGGKKKYKLKHIV; LERFAVNPGLLETSE;
LGQLQPSLQTGSEEL; GSEELRSLTNTVATL; TVATLYCVHQRIEIK;
RIEIKDTKEALDKIE; GPIAPGQMREPRGSD; PRGSDIAGTTSTLQE;
STLQEQIGWMTNNPP; TNNPPIPVGEITKRW; IYKRWIILGLNKIVR;
QGVGGPGHKARVLAE; RVLAEAMSQVTNTAT; EGHTARNCRAPRKKG;
PRKKGCWKCGKEGHQ; KEGHQMKDCTERQAN; WPSYKGRPGNPLQSR;
QEPIDKELYPLTSLR; WRWGWRWGTMLLGML; TEKLWVTVYYGVPVW;
GVPVWKGATTTLFCA; LWDQSLKPCVKLTPL; KLTPLCVSLKCTDLK;
CTDLKNDTNTNSSSG; ISTSIRGKVQKEYAF; KTFNGTGPCTNVSTV;
NVSTVQCTHGIRPVV; IRPVVSTQLLLNGSL; NNTRKSIRIQRGPGR;
GQIRSCCNITGLLLT; SNNESEIFRPGGGDM; IEPLGVAPTKAKRRV;
AKRRVVQREKRAVGI; RAVGIGALFLGFLGA; GFLGAAGSTMGAASM;
GAASMTLTVQARQLL; ARQLLSGIVQQQNNL; QQNNLLRAIEAQQHL;
AQQHLLQLTVWGIKQ; QLLGIWGCSGKCICT; KLICTTAVPWNASWS;
NASWSNKSLEQIWNN; QIWNNMTWMEWDREI; WDREINNYTSLIHSL;
NQQEKNEQELLELDK; LELDKWASLWNWFNI; NWFNITNWLWYIKLF;
YIKLFIMIVGGLVGL; LPIPRGPDRPEGIEE; DRDRSIRLVNGSLAL;
EALKYWWNLLQYWSQ; QYWSQELKNSAVSLL; AVAEGTDRVIEVVQG;
VTNKGRQKVVPLTNT; PLTNTTNQKTELQAI; ELQAIYLALQDSGLE;
VDKLVSAGIRKILFL; LPPVVAKEIVASCDK; ASCDKCQLKGEAMHG;
LEGKVILVAVHVASG; QGVVESMNKELKKII; LKKIIGQVRDQAEHL;
IRDVGKQMAGDDCVA; QMAGDDCVASRQDED; MEPVDPRLEPWKHPG;
MQPIQIAIVALVVAI; LVVAIIIAIVVWSIV; KILRQRKIDRLIDRL;
LIDRLIERAEDSGNE; MEQAPEDQGPQREPH; QREPHNEWTLELLEE;
GLGQHIYETYGDTWA; FLYQSNPPPNPEGTR; QIHSISERILGTYLG;
LTLDCNEDCGTSGTQ; GLHTGERDWHLGQGV; LGQGVSIEWRKKRYS;
KKRYSTQVDPELADQ; ELADQLIHLYYFDCF; KVGSLQYLALAALIT;
AALITPKKIKPPLPS; and PPLPSVTKLTEDRWN in a physiologically-acceptable carrier, said dose being sufficiently large to elicit a serum neutralizing response in an inoculated host.

11. A composition as in claim 10, further comprising a physiologically-acceptable adjuvant.

12. A solid phase immunoassay capable of detecting anti-pathogen antibodies in a patient sample, said immunoassay comprising:
exposing the patient sample to a solid phase having at least two discrete synthetic peptides immobilized thereon, said peptides encoding immunologically distinct epitopes characteristic of the pathogen; and
observing binding between the immobilized peptides and the antibodies in the patient sampl .

13. An immunoassay as in claim 12, wherein the pathogen is HIV-1.

14. A solid phase immunoassay as in claim 13, wherein at least one of said immobilized peptides encodes an epitope characteristic of an HIV-1 regulatory protein.

15. A solid phase immunoassay as in claim 12, having at least four discrete synthetic peptides immobilized thereon.

16. A solid phase immunoassay as in claim 12, having at least eight discrete synthetic peptides immobilized thereon.

17. A solid phase immunoassay as in claim 12, wherein the peptides are chemically synthesized linear fragments.

18. A solid phase immunoassay as in claim 12, wherein the peptides are recombinantly produced fragments.

19. A solid phase immunoassay as in claim 12, wherein the synthetic peptides have a length in the range from about 6 to 50 amino acids.

Claims for the following Contracting State : ES

1. A method for obtaining a peptide having fewer than 100 amino acids and comprising at least one epitope defined by one of the following amino acid sequences:
MGARASVLSGGELDP; GELORWEKIRLRPGG; LRPGGKKKYKLKHIV;
LERFAVNPGLLETSE; LGQLQPSLQTGSEEL; GSEELRSLTNTVATL;
TVATLYCVHQRIEIK; RIEIKDTKEALDKIE; GPIAPGQMREPRGSD;
PRGSDIAGTTSTLQE; STLQEQIGWMTNNPP; TNNPPIPVGEITKRW;
IYKRWIILGLNKIVR; QGVGGPGHKARVLAE; RVLAEAMSQVTNTAT;
EGHTARNCRAPRKKG; PRKKGCWKCGKEGHQ; KEGHQMKDCTERQAN;
WPSYKGRPGNPLQSR; QEPIDKELYPLTSLR; WRWGWRWGTMLLGML;
TEKLWVTVYYGVPVW; GVPVWKGATTTLFCA; LWDQSLKPCVKLTPL;
KLTPLCVSLKCTDLK; CTDLKNDTNTNSSSG; ISTSIRGKVQKEYAF;
KTFNGTGPCTNVSTV; NVSTVQCTHGIRPVV; IRPVVSTQLLLNGSL;
NNTRKSIRIQRGPGR; GQIRSCCNITGLLLT; SNNESEIFRPGGGDM;
IEPLGVAPTKAKRRV; AKRRVVQREKRAVGI; RAVGIGALFLGFLGA;
GFLGAAGSTMGAASM; GAASMTLTVQARQLL; ARQLLSGIVQQQNNL;
QQNNLLRAIEAQQHL; AQQHLLQLTVWGIKQ; QLLGIWGCSGKCICT;
KLICTTAVPWNASWS; NASWSNKSLEQIWNN; QIWNNMTWMEWDREI;
WDREINNYTSLIHSL; NQQEKNEQELLELDK; LELDKWASLWNWFNI;
NWFNITNWLWYIKLF; YIKLFIMIVGGLVGL; LPIPRGPDRPEGIEE;
DRDRSIRLVNGSLAL; EALKYWWNLLQYWSQ; QYWSQELKNSAVSLL;
AVAEGTDRVIEVVQG; VTNKGRQKVVPLTNT; PLTNTTNQKTELQAI;
ELQAIYLALQDSGLE; VDKLVSAGIRKILFL; LPPVVAKEIVASCDK;
ASCDKCQLKGEAMHG; LEGKVILVAVHVASG; QGVVESMNKELKKII;
LKKIIGQVRDQAEHL; IRDVGKQMAGDDCVA; QMAGDDCVASRQDED;
MEPVDPRLEPWKHPG; MQPIQIAIVALVVAI; LVVAIIIAIVVWSIV;
KILRQRKIDRLIDRL; LIDRLIERAEDSGNE; MEQAPEDQGPQREPH;
QREPHNEWTLELLEE; GLGQHIYETYGDTWA; FLYQSNPPPNPEGTR;
QIHSISERILGTYLG; LTLDCNEDCGTSGTQ; GLHTGERDWHLGQGV;
LGQGVSIEWRKKRYS; KKRYSTQVDPELADQ; ELADQLIHLYYFDCF;
KVGSLQYLALAALIT; AALITPKKIKPPLPS; and PPLPSVTKLTEDRWN.

2. A method according to claim 1, wherein the peptide is substantially free from natural folding.

3. A method according to claim 1 or claim 2 wherein the peptide is substantially free from glycosylation.

4. A method of synthesizing a peptide which includes a sequence substantially the same as one selected from:
MGARASVLSGGELDP; GELORWEKIRLRPGG;
LRPGGKKKYKLKHIV; LERFAVNPGLLETSE; LGQLQPSLQTGSEEL;
GSEELRSLTNTVATL; TVATLYCVHQRIEIK; RIEIKDTKEALDKIE;
GPIAPGQMREPRGSD; PRGSDIAGTTSTLQE; STLQEQIGWMTNNPP;
TNNPPIPVGEITKRW; IYKRWIILGLNKIVR; QGVGGPGHKARVLAE;
RVLAEAMSQVTNTAT; EGHTARNCRAPRKKG; PRKKGCWKCGKEGHQ;
KEGHQMKDCTERQAN; WPSYKGRPGNPLQSR; QEPIDKELYPLTSLR;
WRWGWRWGTMLLGML; TEKLWVTVYYGVPVW; GVPVWKGATTTLFCA;

LWDQSLKPCVKLTPL; KLTPLCVSLKCTDLK; CTDLKNDTNTNSSSG; ISTSIRGKVQKEYAF; KTFNGTGPCTNVSTV; NVSTVQCTHGIRPVV; IRPVVSTQLLLNGSL; NNTRKSIRIQRGPGR; GQIRSCCNITGLLLT; SNNESEIFRPGGGDM; IEPLGVAPTKAKRRV; AKRRVVQREKRAVGI; RAVGIGALFLGFLGA; GFLGAAGSTMGAASM; GAASMTLTVQARQLL; ARQLLSGIVQQQNNL; QQNNLLRAIEAQQHL; AQQHLLQLTVWGIKQ; QLLGIWGCSGKCICT; KLICTTAVPWNASWS; NASWSNKSLEQIWNN; QIWNNMTWMEWDREI; WDREINNYTSLIHSL; NQQEKNEQELLELDK; LELDKWASLWNWFNI; NWFNITNWLWYIKLF; YIKLFIMIVGGLVGL; LPIPRGPDRPEGIEE; DRDRSIRLVNGSLAL; EALKYWWNLLQYWSQ; QYWSQELKNSAVSLL; AVAEGTDRVIEVVQG; VTNKGRQKVVPLTNT; PLTNTTNQKTELQAI; ELQAIYLALQDSGLE; VDKLVSAGIRKILFL; LPPVVAKEIVASCDK; ASCDKCQLKGEAMHG; LEGKVILVAVHVASG; QGVVESMNKELKKII; LKKIIGQVRDQAEHL; IRDVGKQMAGDDCVA; QMAGDDCVASRQDED; MEPVDPRLEPWKHPG; MQPIQIAIVALVVAI; LVVAIIIAIVVWSIV; KILRQRKIDRLIDRL; LIDRLIERAEDSGNE; MEQAPEDQGPQREPH; QREPHNEWTLELLEE; GLGQHIYETYGDTWA; FLYQSNPPPNPEGTR; QIHSISERILGTYLG; LTLDCNEDCGTSGTQ; GLHTGERDWHLGQGV; LGQGVSIEWRKKRYS; KKRYSTQVDPELADQ; ELADQLIHLYYFDCF; KVGSLQYLALAALIT; AALITPKKIKPPLPS; and PPLPSVTKLTEDRWN.

5. A method for synthesizing a peptide as defined in claim 1 or claim 4, wherein the peptide is substantially free from natural folding.

6. A method of synthesizing a peptide according to claim 4, wherein the peptide is substantially free from glycosylation.

7. A method for preparing an antibody reactive with at least one epitope defined by the following amino acid sequences:
MGARASVLSGGELDP; GELQRWEKIRLRPGG; LRPGGKKKYKLKHIV; LERFAVNPGLLETSE; LGQLQPSLQTGSEEL; GSEELRSLTNTVATL; TVATLYCVHQRIEIK; RIEIKDTKEALDKIE; GPIAPGQMREPRGSD; PRGSDIAGTTSTLQE; STLQEQIGWMTNNPP; TNNPPIPVGEITKRW; IYKRWIILGLNKIVR; QGVGGPGHKARVLAE; RVLAEAMSQVTNTAT; EGHTARNCRAPRKKG; PRKKGCWKCGKEGHQ; KEGHQMKDCTERQAN; WPSYKGRPGNPLQSR; QEPIDKELYPLTSLR; WRWGWRWGTMLLGML; TEKLWVTVYYGVPVW; GVPVWKGATTTLFCA; LWDQSLKPCVKLTPL; KLTPLCVSLKCTDLK; CTDLKNDTNTNSSSG; ISTSIRGKVQKEYAF; KTFNGTGPCTNVSTV; NVSTVQCTHGIRPVV; IRPVVSTQLLLNGSL; NNTRKSIRIQRGPGR; GQIRSCCNITGLLLT; SNNESEIFRPGGGDM; IEPLGVAPTKAKRRV; AKRRVVQREKRAVGI; RAVGIGALFLGFLGA; GFLGAAGSTMGAASM; GAASMTLTVQARQLL; ARQLLSGIVQQQNNL; QQNNLLRAIEAQQHL; AQQHLLQLTVWGIKQ; QLLGIWGCSGKCICT; KLICTTAVPWNASWS; NASWSNKSLEQIWNN; QIWNNMTWMEWDREI; WDREINNYTSLIHSL; NQQEKNEQELLELDK; LELDKWASLWNWFNI; NWFNITNWLWYIKLF; YIKLFIMIVGGLVGL; LPIPRGPDRPEGIEE; DRDRSIRLVNGSLAL; EALKYWWNLLQYWSQ; QYWSQELKNSAVSLL; AVAEGTDRVIEVVQG; VTNKGRQKVVPLTNT; PLTNTTNQKTELQAI; ELQAIYLALQDSGLE; VDKLVSAGIRKILFL; LPPVVAKEIVASCDK; ASCDKCQLKGEAMHG; LEGKVILVAVHVASG; QGVVESMNKELKKII; LKKIIGQVRDQAEHL; IRDVGKQMAGDDCVA; QMAGDDCVASRQDED; MEPVDPRLEPWKHPG; MQPIQIAIVALVVAI; LVVAIIIAIVVWSIV; KILRQRKIDRLIDRL; LIDRLIERAEDSGNE; MEQAPEDQGPQREPH; QREPHNEWTLELLEE; GLGQHIYETYGDTWA; FLYQSNPPPNPEGTR; QIHSISERILGTYLG; LTLDCNEDCGTSGTQ; GLHTGERDWHLGQGV; LGQGVSIEWRKKRYS; KKRYSTQVDPELADQ; ELADQLIHLYYFDCF; KVGSLQYLALAALIT; AALITPKKIKPPLPS; and PPLPSVTKLTEDRWN.

8. A method for preparing a monoclonal antibody reactiv with at least one epitope as defined in claim 7.

9. A method for preparing a polyclonal antibody reactiv with at least on epitope as defined in claim 7.

10. A process for preparing a composition for eliciting an immune response against HIV-1 infection, said process comprising combining a dose of a peptide including a sequence substantially the same as on selected from:
MGARASVLSGGELDP;
GELQRWEKIRLRPGG; LRPGGKKKYKLKHIV; LERFAVNPGLLETSE;
LGQLQPSLQTGSEEL; GSEELRSLTNTVATL; TVATLYCVHQRIEIK;
RIEIKDTKEALDKIE; GPIAPGQMREPRGSD; PRGSDIAGTTSTLQE;
STLQEQIGWMTNNPP; TNNPPIPVGEITKRW; IYKRWIILGLNKIVR;
QGVGGPGHKARVLAE; RVLAEAMSQVTNTAT; EGHTARNCRAPRKKG;
PRKKGCWKCGKEGHQ; KEGHQMKDCTERQAN; WPSYKGRPGNPLQSR;
QEPIDKELYPLTSLR; WRWGWRWGTMLLGML; TEKLWVTVYYGVPVW;
GVPVWKGATTTLFCA; LWDQSLKPCVKLTPL; KLTPLCVSLKCTDLK;
CTDLKNDTNTNSSSG; ISTSIRGKVQKEYAF; KTFNGTGPCTNVSTV;
NVSTVQCTHGIRPVV; IRPVVSTQLLLNGSL; NNTRKSIRIQRGPGR;
GQIRSCCNITGLLLT; SNNESEIFRPGGGDM; IEPLGVAPTKAKRRV;
AKRRVVQREKRAVGI; RAVGIGALFLGFLGA; GFLGAAGSTMGAASM;
GAASMTLTVQARQLL; ARQLLSGIVQQQNNL; QQNNLLRAIEAQQHL;
AQQHLLQLTVWGIKQ; QLLGIWGCSGKCICT; KLICTTAVPWNASWS;
NASWSNKSLEQIWNN; QIWNNMTWMEWDREI; WDREINNYTSLIHSL;
NQQEKNEQELLELDK; LELDKWASLWNWFNI; NWFNITNWLWYIKLF;
YIKLFIMIVGGLVGL; LPIPRGPDRPEGIEE; DRDRSIRLVNGSLAL;
EALKYWWNLLQYWSQ; QYWSQELKNSAVSLL; AVAEGTDRVIEVVQG;
VTNKGRQKVVPLTNT; PLTNTTNQKTELQAI; ELQAIYLALQDSGLE;
VDKLVSAGIRKILFL; LPPVVAKEIVASCDK; ASCDKCQLKGEAMHG;
LEGKVILVAVHVASG; QGVVESMNKELKKII; LKKIIGQVRDQAEHL;
IRDVGKQMAGDDCVA; QMAGDDCVASRQDED; MEPVDPRLEPWKHPG;
MQPIQIAIVALVVAI; LVVAIIIAIVVWSIV; KILRQRKIDRLIDRL;
LIDRLIERAEDSGNE; MEQAPEDQGPQREPH; QREPHNEWTLELLEE;
GLGQHIYETYGDTWA; FLYQSNPPPNPEGTR; QIHSISERILGTYLG;
LTLDCNEDCGTSGTQ; GLHTGERDWHLGQGV; LGQGVSIEWRKKRYS;
KKRYSTQVDPELADQ; ELADQLIHLYYFDCF; KVGSLQYLALAALIT;
AALITPKKIKPPLPS; and PPLPSVTKLTEDRWN and a physiologically-acceptable carrier, said does being sufficiently large to elicit a serum neutralizing response in an inoculated host.

11. A process according to claim 10, wherein a physiologically-acceptable adjuvant is also combined with the dose and the carrier.

12. A solid phase immunoassay capable of detecting anti-pathogen antibodies in a patient sample, said immunoassay comprising:
exposing the patient sample to a solid phase having at least two discrete synthetic peptides immobilized thereon, said peptides encoding immunologically distinct epitopes characteristic of the pathogen; and
observing binding between the immobilized peptides and the antibodies in the patient sample.

13. An immunoassay as in claim 12, wherein the pathogen is HIV-1.

14. A solid phase immunoassay as in claim 13, wherein at least one of said immobilized peptides encodes an epitope characteristic of an HIV-1 regulatory protein.

15. A solid phase immunoassay as in claim 12, having at least four discrete synthetic peptides immobilized thereon.

16. A solid phase immunoassay as in claim 12, having at least eight discrete synthetic peptides immobilized thereon.

17. A solid phase immunoassay as in claim 12, wherein the peptides are chemically synthesized linear fragments.

18. A solid phase immunoassay as in claim 12, wherein the peptides are recombinantly produced fragments.

19. A solid phase immunoassay as in claim 12, wherein the synthetic peptides have a length in the range from about 6 to 50 amino acids.

File

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)


(51) International Patent Classification 6 : A01H 4/00, A61K 39/00, C07K 14/00 — A1

(11) International Publication Number: **WO 98/08375**

(43) International Publication Date: 5 March 1998 (05.03.98)

(21) International Application Number: PCT/US97/15200

(22) International Filing Date: 28 August 1997 (28.08.97)

(30) Priority Data:
08/704,856 28 August 1996 (28.08.96) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/704,856 (CIP)
Filed on 28 August 1996 (28.08.96)



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(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*


(54) Title: POLYPEPTIDES FUSED WITH ALFALFA MOSAIC VIRUS OR ILARVIRUS CAPSID


(57) Abstract

A fusion capsid protein comprising a plant virus capsid protein fused to an antigenic polypeptide is used as a molecule for presentation of that polypeptide to the immune system of an animal such as a human. The plant virus capsid protein is that of an alfalfa mosaic virus (AlMV) or ilarvirus.

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# POLYPEPTIDES FUSED WITH ALFALFA MOSAIC VIRUS OR ILARVIRUS CAPSID

## FIELD OF THE INVENTION

The field of the invention is recombinant plant viruses, especially their use as immunizing agents which carry antigenic sequences from mammalian (e.g., human) or other animal pathogens and their use as a system for increased production of polypeptides of interest.

## BACKGROUND OF THE INVENTION

Traditionally, successful vaccination has been dependent upon the use of live attenuated viruses or preparations of killed pathogenic organisms. These vaccines are very effective in controlling or, as in the case of smallpox, even eliminating certain infectious diseases. However, their use often present safety concerns. Subunit vaccines based on peptide or proteins derived from a pathogen are less hazardous than traditional vaccines but have generally suffered from poor immunogenicity and high expense. Moreover, current vaccines with a few exceptions must be administered parenterally. However, it is well known that most pathogens gain entry across the mucosal surfaces of the body and a mucosal immune response would therefore be more appropriate.

Both safety concerns and the desire to target mucosal tissues for more effective immunization against common pathogens dictate the need for new approaches to vaccination.

For induction of a mucosal response, oral administration of antigen is appropriate, inexpensive, and safe. However, in order to efficiently immunize by the oral route, several obstacles such as degradation from low pH or proteases in the gastrointestinal GI tract, the short exposure to immune induction sites, and limited permeability must be overcome.

Recent studies demonstrate that plants and plant viruses can function as effective tools for vaccine production and delivery. Furthermore, like liposomes and microcapsules, it is expected that plant cells and plant viruses will serve as delivery vehicles providing natural protection for the antigen associated with them and enhancing the uptake of the antigen from the GI tract. Such new developing "green system vaccines" have significant advantages over the traditional and synthetic vaccines as regards safety, deliverability via either parenteral, nasal or oral routes, and lower cost of production.

## BRIEF SUMMARY OF THE INVENTION

In one general aspect, the current invention is a process of delivering a fusion capsid protein (a plant virus capsid protein fused to a foreign polypeptide) to a mammal (such as a human) or other animal using recombinant tobacco mosaic virus (TMV) genetic material (TMV genetic material combined with genetic material that codes for a chimeric capsid protein, the chimeric capsid protein being capsid protein of either an alfalfa mosaic virus (AlMV) capsid protein (CP) or ilarvirus CP, fused to the foreign polypeptide) as a delivery

vehicle to that mammal or other animal. A foreign polypeptide is one that does not naturally occur in either TMV, an AlMV or an ilarvirus. The fusion protein is administered to the mammal or other animal for purposes of inducing an immune response against the foreign polypeptide. In a second general aspect, the invention is a production process: the use of such a chimeric virus to express the fused coat protein (comprising either an antigenic or nonantigenic foreign protein) in a plant.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1A.** Schematic representation of the genome of TMV. The regions of the genome coding for the 126 kDa and 183 kDa proteins required for virus replication, the 30 kDa viral movement protein, and the CP (viral coat protein) are shown schematically. The arrow under "TMV CP SP" indicates the subgenomic promoter of TMV. The three connected ellipsoids under "pep" represent the polypeptide fused to the AlMV CP. Rz- indicates ribozyme. B3ORz- is a derivative of TMV. AvB3ORz- is a derivative of B3ORz and is defective as to translation of coat protein.

**Figure 1B.** A schematic representation of cloning strategy: the cloning of chimeric AlMV CP into a TMV based vector. The sequences of polypeptides from HIV-1 and rabies virus are used as the "pep" to create recombinant viruses.

The "pep" polypeptide has the following amino sequence: For BrzCPMNV3: CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC (SEQ ID NO:1)

For BRzCPNLV3: CTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHC (SEQ ID NO:2)

For BRzCPDnv10c: MSAVYTRIMMNGGRLKRYEAAELTLTDVALADDS (SEQ ID NO:3)

For BRzCPDrg24: MSAVYTRIMMNGGRLKRPPDQLVALHDGIEKLVVEEDS (SEQ ID NO:4)

For BRzCPNLpr:

HIV-1 NL 4.3 Vpr-
MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTW
AGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS
(SEQ ID NO:15)

For BRzCPNLVpu:

HIV-1 NL 4.3 Vpu-
MQPIIVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGN
ESEGEVSALVEMGVEMGHHAPWDIDDL (SEQ ID NO:16)

**Figure 2**. Accumulation of chimeric AlMV CP, fused with different peptides, in tobacco protoplasts infected with transcripts of recombinant virus. Proteins were separated by electrophoresis in a 13% SDS-polyacrylamide gel and electroblotted on nylon membrane. The proteins were reacted with monoclonal antibodies to AlMV CP followed by detection with Westatin immunostain kit (Sigma). Lane 1 represents wt AlMV CP. Lane 2 and 3 are *in vitro* translation products of pSPCPD10c and pSPCPDrg24, respectively. Lane 4- pBRzCPNLVpu, lane 5-pBRzCPNLVpr, lane 6-pBRzCPDrg24, lane 7-pBRzCPDNV10c, lane 8-pBRzCPMNV3, lane 9-pBRzCPNLV3, and lane 10-B30Rz.

**Figure 3**. Electron micrographs of recombinant AlMV particles from tobacco plants, infected with recombinant transcripts of TMV presenting different constructs. The

particles were negatively stained with 2% of uranyl acetate. The bars indicate 100 nm. A-B30Rz. B- pBRzCPNLV3. The single arrow indicates a TMV particle. The double arrow indicates indicates recombinant AlLMV particles.

**Figure 4**. Accumulation of chimeric AIMV CP in systemically infected tobaccco leaves. The tobacco leaves were inoculated with transcripts of recombinant virus. Proteins were separated by electrophoresis in a 13% SDS-polyacrilamide gel. The proteins were reacted with monoclonal antibodies to AIMV CP followed by detection with Westatin immunostain kit. Lane 1 represents wt AIMV CP. Two- pBRzCPNLVpu, 3- B30Rz, 4- pBRzCPDrg24, 5- pBRzCPDNV10c, 6- pBRzCPNLVpr, 7- pBRzCPMNV3, and 8- pBRzCPNLV3.

**Figure 5**. Immunoprecipitation of chimeric particles containing rabies and HIV-1 epitodes. The particles purified from plant tissue which were coinfected with transcripts of recombinant virus and immunoprecipitated using monoclonal antibodies to the linear epitope of rabies G protein (rg24). Immunoprecipitated proteins were separated by electrophoresis in a 13% SDS-polyacrilamide gel. The proteins were reacted with antibodies to HIV-1 V3 loop or with monoclonal antibodies to N protein of rabies followed by detection with Westatin immunostain kit. Lane 1 represents immunoprecipitated proteins reacting with antibodies to N protein. Two- wild type rabies. 3- HIV-1 gp120 reacted with antibodies to V3 loop, and 4- immunoprecipitated proteins reacting with antibodies to V3 loop.

**Figure 6A** and **B**. Results obtained in ELISA and neutralization assay as a comparison of control mouse immunized with mix of TMV and AIMV and mouse immunized with pBRzCPDrg24. Eight-week-old female Swiss-Webster outbred mice were immunized with 10-ug doses of each recombinant virus engineered to express epitopes. Three immunizations of 0.1 mL were administered intraperitoneally at 2-week intervals with complete Freund's adjuvant at a 1:1 (v:v) ratio. An equal quantity of a mixture of wild type TMV plus AIMV was used with complete Freund's adjuvant as a control. Ten to 14 days after each immunization, serum samples were obtained from individual mice, and specific antibody titers for rabies virus were assessed using a solid-phase enzyme-linked immunosorbent assay. Assay results are expressed in O.D. units. Specific neutralization of rabies CVS-11 virus was assessed in a modified rapid fluoresecent focus-forming assay using serum from pBRzCPDrg24-immunized mice and BHK indicator cells. One out of five mice had neutralizing antibodies.

**Figure 7**. The precentage of mice producing specific rabies virus antibodies after I. p. immunization with pBRzCPDrg24 (Rg24-A/TMV) or mix of AIMV plus TMV (Control). The results were obtained in ELISA. Experiment is described in the legend to **Figure 6**.

**Figure 8**. Serum antibody response of mice immunized with CPMNV3 and neutralizing activity of those antibodies. Serum antibody response was measured by ELISA on plates coated with synthetic peptide resembling the V3 loop of HIV-1. **Fig. 8A** indicates the serum antibody response when the immunogen

was administered without CFA whereas in **Fig. 8B** CFA was added. Also shown are the ELISA data for preimmune and mice immunized with control virus. **Fig. 8C** represents neutralization of HIV-1/MN isolate by sera from mice immunized with CPMNV3. Data points in **Fig. 7C** are averages obtained using preimmune and sera after the last (seventh) inoculation of antigen.

**Figure 9.** Serum antibody response of mice immunized with CPDrg24. Serum antibody response was measured by ELISA using a 1:40 dilution of serum and plates coated with AlMV (vector plates) or inactivated ERA strain rabies virus (ERA plates). **Fig. 9A** shows the serum IgA response specific for vector and Drg24 whereas in **Fig. 9B** shows the serum IgG response specific for vector and Drg24. Also shown are the ELISA data for preimmune and mice immunized with control virus.

## DETAILED DESCRIPTION

### GLOSSARY AND DISCUSSION OF TERMS USED

A "plant" for purposes of this patent application includes liverworts (Hepaticae), mosses (Musci), psilopsids (Psilopsida), club mosses (Lycopsida), horsetails (Sphenopsida), ferns and seed plants, and certain fungi specified below, and algae including blue-green algae. Ferns and seed plants together make up the Pteropsida. Seed plants include gymnosperms (Gymnospermae) and angiosperms (Angiospermae). The great majority of plants used for food are angiosperms. For purposes of this application, the following fungi are considered plants: Basidiomycetes, which include

mushrooms. The following are not considered plants for purposes of this application: bacteria, single-celled eukaryotes, and the following fungi: Phycomycetes, Ascomycetes (yeasts), and Deuteromycetes.

The term "plant tissue" includes any tissue of a plant. Included are whole plants, any part of plants, plant cells, plants seeds, and plant protoplasts.

The word "animal" refers to humans as well as other animals.

A "bird" is a warm-blooded vertebrate of the class Aves.

A "fish" is a cold-blooded aquatic vertebrate, having gills and fins.

A "chimeric protein" is created when two or more genes that normally code for two separate proteins recombine, either naturally or as the result of human intervention, to code for a protein that is a combination of all or part of each of those two proteins.

The phrase "code for" is used in this application to refer to both the nucleic acid sequence that codes for a polypeptide sequence and a base sequence complementary to such a nucleic acid sequence.

A "fusion capsid protein" is a chimeric protein in which one of the genes in the chimera codes for a plant virus capsid protein.

A "bipartite fusion capsid protein" is a fusion wherein genes for two proteins recombine.

A "tripartite fusion capsid protein" is a fusion wherein genes for three proteins recombine.

A "pathogen protein" is a protein that is coded for by the genetic material of a pathogen.

A "naturally occurring plant protein" is one that is normally found in a plant, at least one stage in its life cycle, in its natural habitat.

"Infecting a plant cell" means infecting a plant cell with one or more genes that it does not naturally have, the infection being by a nucleic acid molecule that may or may not be encapsidated within a virus.

"Protective immunity" is the ability of an animal, such as a mammal, bird, or fish, to resist (delayed onset of symptoms or reduced severity of symptoms), as the result of its exposure to the antigen of a pathogen, disease or death that otherwise follows contact with the pathogen. Protective immunity is achieved by one or more of the following mechanisms: mucosal, humoral, or cellular immunity. Mucosal immunity is primarily the result of secretory IgA (sIGA) antibodies on mucosal surfaces of the respiratory, gastrointestinal, and genitourinary tracts. The sIGA antibodies are generated after a series of events mediated by antigen-processing cells, B and T lymphocytes, that result in sIGA production by B lymphocytes on mucosa-lined tissues of the body. Mucosal immunity can be stimulated by an oral vaccine. The primary result of protective immunity is the destruction of the pathogen or inhibition of its ability to replicate itself.

"Humoral immunity" is the result of IgG antibodies and IgM antibodies in serum.

"Cellular immunity" can be achieved through cytotoxic T lymphocytes or through delayed-type hypersensitivity that involves macrophages and T lymphocytes, as well as other mechanisms involving T cells without a requirement for antibodies. A "derivative cell" derived from an infected plant cell is one created as a result of the infected plant cell undergoing cell division or a series of cell divisions such that one or more copies of the foreign gene introduced into the plant cell by infection is in the derivative cell.

"Ilarviruses" includes the following subgroups: tobacco streak virus, prune dwarf virus, lilac ring mottle virus, citrus leaf rugose virus, citrus variegation virus, elm mottle virus, spinach latent virus, asparagus virus 2, Parietaria mottle virus, hydrangea mosaic virus, apple mosaic virus, Prunus necrotic ringspot virus, tulare apple mosaic virus, blueberry scorch virus, cherry rugose virus, danish plum line pattern virus, Hop A virus, Hop C virus, American plum line pattern virus, and Humulus japonicus virus.

A "recombinant virus" is one in which the genetic material of a virus has combined with other genetic material.

A "polypeptide" is a molecule in which there is at least four amino acids linked by peptide bonds.

"Viral nucleic acid" may be the genome (or the majority thereof) of a virus, or a nucleic acid molecule complementary in base sequence to that genome. A DNA molecule that is complementary to viral RNA is also considered viral nucleic acid. An RNA molecule that is complementary in base sequence to viral DNA is also considered to be viral nucleic acid.

"AlMV" is alfalfa mosaic virus.

"TMV" is tobacco mosaic virus.

A "vaccine" in the present invention is the fusion capsid protein, any particle of which that protein is part, or any preparation such as plant material of which that protein is part.

"Plurality" means more than one.

ASPECTS OF THE INVENTION

In a general aspect, the invention is a process of administering a polypeptide to an animal (especially a mammal, bird, or fish), the process comprising the steps of:

(1) infecting a plant cell with recombinant plant virus nucleic acid that will be processed in the plant cell to produce a fusion capsid protein comprising virus capsid protein and a polypeptide that is not a plant virus capsid protein, said virus capsid protein being an AlMV coat protein or ilarvirus capsid protein, thereby creating a infected cell;

(2) cultivating the infected cell, or a derivative cell derived from said infected cell, under conditions where said infected cell or derivative cell makes the fusion capsid protein; and

(3) administering the fusion capsid protein or a portion thereof to an animal.

In step (1) the viral nucleic acid may either be in a virus or not in a virus. If it is not in a virus, it may either be pure nucleic acid or nucleic acid associated with other molecules or molecular structures.

In step (1) if the recombinant plant virus nucleic acid comprises TMV nucleic acid, the TMV nucleic acid will preferably comprise nucleic acid coding for the TMV 123 kDA 183 kDa, and 30 kDa proteins.

In steps (1) and (2), the infected cell or derivative cell may either be part of a plant or plant tissue or may be free of other plant cells.

In step (1), it is preferable that the viral nucleic acid have sufficient genetic information to produce viral particles during step (2).

The route of administration in step (3) can be parenteral or nonparenteral. If administered parenterally, the protein to be administered to the animal will preferably be substantially pure of other material found in the plant cells that produced it .

In step (3), the protein or portion thereof may be in or part of the infected or derivative cell, part of an extract of such a infected or derivative cell or, as a result of protein purification, free of other material normally present in the infected or derivative cell.

The result of step (3) is, in a particular embodiment of the invention, an immune response against the part of the fusion capsid protein that is not plant viral protein. That immune response preferably results in either protective immunity or systemic tolerance.

The steps for purifying a fusion capsid protein are ones commonly used for the fractionation of plants into their protein components and the separation of individual proteins

from other components of the infected or derivative cell. Such steps include protection of the native conformation of the pathogen protein by steps such as flash freezing the plant material with dry ice or liquid nitrogen.

An extract would be created by a process comprising mechanical or chemical disruption of a cell. In some cases, additional protein purification steps would be used to make the extract.

In one important embodiment of the invention, in step (3) the fusion capsid protein is in part of a plant or plant product and is fed to the mammal (i.e. oral route of administration). In such a case, it is preferable that the plant is raw; i.e., has not been cooked (heated above the temperatures associated with growth, storage, and transport). In the examples described below, the plant is not cooked. Animals typically may eat the plant, pieces of the plant, a puree from the plant, or plant juice. As a result, it is frequently preferred that step (2) takes place in an edible plant or part of an edible plant.

Animals vary as regards which food is edible. Plants of greatest interest include potatoes, tomatoes, peas, beans, alfalfa, citrus fruits (e.g., oranges, lemons, grapefruit), grapes, carrots, strawberries, blueberries and other berries, bananas, rice, wheat, corn, barley, oats, rye, dates, cabbage, Brussel sprouts, cauliflower, turnips, cucurbits, papaya, guava, apples, cherries, apricots, pears, and grapes.

In another important embodiment of the invention, the fusion capsid protein of interest is extracted in purified

form from the plant and administered as a substantially pure protein, possibly with an adjuvant or other compounds needed to facilitate or improve vaccine administration.

As in the case of the rabies virus glycoprotein (Rgp), the polypeptide referred to in step (1) may be one that is a glycoprotein in the pathogen.

**Pathogens of interest**

A pathogen is any organism such as a virus, bacterium, fungus, or parasite, as well as a protein which is capable of self-replication such as a prion and capable of inducing disease in an animal.

Pathogens against which vaccines created by the present invention are effective are those including but not limited to bacteria of the genera streptococci and staphylococci, as well as the mycoplasma, rickettsia and spirochetes. The following viral groups of the parvoviridae, papovaviridae, adenoviridae, herpesviridae, poxviridae, iridoviridae, picornaviridae, caliciviridae, togaviridae, flaviviridae, coronaviridae, ortho- and paramyxoviridae, rhabdoviridae, bunyaviridae, reoviridae, birnaviridae, and the retroviruses all contain representative members with application for vaccines of the present invention.

Pathogens against which vaccines of the present invention are expected to be particularly useful are rabies, respiratory syncytial virus, cholera, typhoid fever, herpes simplex types I and II, tuberculosis, pathogenic pneumococci, human immunodeficiency virus-1 (HIV-1) and human immunodeficiency

virus-2 (HIV-2). The spectrum of pathogens includes those of veterinary significance and includes parvoviridae, papovaviridae, adenoviridae, herpesviridae, poxviridae, iridoviridae, picornaviridae, caliciviridae, togaviridae, flaviviridae, coronaviridae, ortho- and paramyxoviridae, rhabdoviridae, bunyaviridae, reoviridae, birnaviridae, and the retroviruses. Both gram negative and gram positive bacteria and spirochetes are also expected to be pathogens which may be clinically affected by the plant vaccines of the present invention.

**Methods for infecting plants**

Genes may be transferred by any of a variety of infection means, which means include but are not limited to:

1) Coating a plant surface with viruses or viral nucleic acid, possibly one that has undergone abrasion;

2) Microparticle bombardment, for example as described for wheat by V. Vasil et al., *Bio/Technology* 9, 743 (1991) and generally in *Bio/Technology* 10, 286 (1992).

3) Electroporation, for example as described for lettuce by M. C. Chupeau et al., *Bio/Technology* 7, 503 (1989).

4) Liposome fusion with protoplasts (A. Deshayes et all., *EMBO J.* 4, p2731-2737 (1985)).

5) Polyethylene glycol-mediated transformation (I. Potrykus et al., *Mol. Gen Genetics*, 197, 183-188).

6) Microinjection (R. Griesbach, Biotechnology 3, p348-350; C.K. Shewmaker *Mol. Gen. Genetics*, 202 p. 179-185 (1986)).

It may be desirable to target protein expression to different plant tissues (i.e. fruit and leaves) and to different intracellular locations (i.e. chloroplast, vacuole, plasma membrane) in order to enhance protection of the recombinant protein. It may also be desirable to present the antigen as part of larger structure for uptake by the M cells of the Peyers patch.

It may be desirable to combine presentation of the primary antigens with a biologically active molecule that will stimulate and or enhance an immune response and serve as an adjuvant. Since modified plants can be crossed genetically, plants expressing an adjuvant protein and a primary antigen can be produced separately and combined to deliver antigen and adjuvant in one plant.

**Plant viruses**

The quasi-isometric/bacilliform viruses can, according to J. A. Levy et al. (Virology, 3rd edition, Prentice Hall, Englewood Cliffs, New Jersey (1994)), be divided into two subgroups: AlMV and the ilarviruses. The antigen-presenting capsid protein in the present invention is that of a quasi/bacilliform virus: its synthesis is driven by a genome constructed to produce a chimeric capsid protein whose components include the quasi/bacilliform capsid protein and the antigenic protein or polypeptide of interest. The virus that delivers the chimeric capsid protein to a plant for production of the antigenic protein may itself be a quasi-isometric/bacilliform virus, or alternatively, may be a

nonenveloped virus with a positive-sense RNA genome that is not a quasi-isometric/bacilliform virus. Below, examples are given of how AlMV or TMV can form the major part of the delivery vehicle.

**Tobacco mosaic virus and alfalfa mosaic virus**

Two viruses used herein to exemplify the invention are tobacco mosaic virus (TMV) and alfalfa mosaic virus (AlMV). TMV is the type member of the tobamovirus group. TMV consists of a single plus-sense genomic RNA (6.5 kb) encapsidated with a unique coat protein (17.5 kDa) which results in rod-shaped particles (300 nm). A wide host range of tobacco mosaic virus allows one to use a variety of plant species as production and delivery systems. The expression of protein depends only upon virus replication and does not interfere with plant nuclear transcription machinery thus providing high levels of protein accumulation in infected cells. It has been shown that foreign genes inserted into this vector can produce high levels of protein (Yusibov et al., *Proc. Natl. Acad. Sci. U.S.* 92, 8980 (1995)). The second component of a recombinant expression vector exemplified herein is AlMV CP. AIMV is a type member of *Bromoviridae* family. The genome of this virus consists of three positive-sense RNAs (RNAs 1-3) which are encapsidated by CP (24 kDa) resulting in a 30-60 nm bacilliform particle. The fourth RNA (subgenomic RNA4) of AlMV is the messenger for CP and is synthesized from genomic RNA3. RNA4 is separately encapsidated into spherical particles of 20, 30 nm. The

functions of CP thought to be important for the virus include: initiation of infection, stability of viral RNA, switching from minus to plus RNA, movement of the infectious material throughout the host, and encapsulation. The N-terminus of the AlMV CP specifically binds to the 3' noncoding region of all AlMV RNAs, requiring the AUGC repeats at the 3' end of viral RNAs and initiates the infection. The carboxyterminus of CP is required for the encapsidation of viral RNA. Earlier we have shown that AlMV CP carrying 37 amino acids at its N-terminus will preserve the biological activity and form the particles *in vitro* (Yusibov et *al.*, J. Gen. Virol 77, 567 (1996)). Because of the ability of AlMV CP to form these particles *in vitro* and carry large peptides, the AlMV CP is used as an additional component to complement TMV expression system. *In vitro* transcripts of TMV carrying inserted sequences are used for the mechanical inoculation of tobacco plants. Within 10-14 days after inoculation, infected plant tissue can be used to determine the amount of protein in tissue, for purification of virus particles, and for the feeding experiments to test mucosal immunogenicity. For the latter, low alkaloid tobacco plants that can be fed to the animals are used to grow the recombinant viruses.

After infection with recombinant viral RNA coding for a fusion protein (AIMV capsid protein fused to an antigenic polypeptide), the infecting RNA is processed and the infection results in two types of viral particles:

1) recombinant TMV-recombinant viral RNA encapsidated with wild type TMV CP;

2) recombinant AlMV-non infectious AlMV particles consisting of subgenomic RNA coding for fusion protein (AlMV capsid protein fused to an antigenic polypeptide) and fusion protein.

The particles consisting of recombinant AlMV CP serve as antigen delivery and presentation systems. These particles have advantages over recombinant TMV particles that do not have AlMV or ilarvirus fusion capsid proteins. The use of AlMV or ilarvirus capsid protein allows the assembly of up to 100 amino acids presenting foreign antigenic or nonantigenic sequences into particles for effective antigen presentation.The TMV CP is capable presenting up to 25 amino acids. The larger peptides interferes with TMV assembly. Additionally, AlMV CP molecules each of which is fused to different antigenic polypeptides will assemble into multivalent particles carrying multiple antigens, which allows simultaneous immunization against multiple pathogens.

**EXAMPLES**

EXAMPLE 1

**Construction of Vectors and Method of Plant Transformation**

Construction of Plasmids containing recombinant TMV carrying chimeric AlMV CP Tobacco mosaic virus was used as a vector for the expression of chimeric genes. The plasmids B30Rz and AvB30Rz (Proc. Natl. Acad. Sci. U.S. 88, 7204 (1991)) containing the TMV genome and multiple cloning sites were a gift from Dr. William Dawson of Florida University. All

fusion capsid proteins were made using AlMV CP where the first AUG codon (start codon for *in vivo* translation of AlMV CP) was exchanged for TCG to create an *XhoI* (CTCGAG) site for cloning and an RNA molecule defective in translation (an RNA molecule which does not have a continuous open reading frame that will support the synthesis of stable and detectible polypeptide *in vivo* or *in vitro*, pSPΔAUG, (Yusibov and Loesch-fries, Virology 208, 405 (1994)). For example, Peptides or proteins from human immunodeficiency virus (HIV) and from rabies virus were engineered as fusion with capsid protein of AlMV and cloned into B30Rz.

Construction of fusion protein consisting of full length AlMV CP and V3 loop of HIV-1MN strain

A plasmid DNA containing sequences for HIV-1 envelope protein (*env* ,160) was used as a template for the polymerase chain reaction (PCR). (Such a plasmid can be made by cloning the cDNA of the HIV-1 MN strain containing gp120 sequences including the V3 loop by using PCR cloning into the PCRII vector (Invitrogen, Inc.) or other appropriate vector. The plasmid used here was supplied by David Weiner of U. Pennsylvania). PCR on the plasmid DNA containing sequences for HIV-1 envelope protein was performed using 5'-AGATCTCGAGATGAGTTCATCTGTAGAAATTAATTGTACA-3' as the first strand- and 5'-CGGCTCGAGCTACTAATGTTACAATG-3' as the second strand primers. The PCR products were digested by *XhoI* and ligated into pSPCPΔAUG linearized by *XhoI* . The ligation product, pSPCPMNV3, contained the DNA coding for the HIV V3

loop and full length AlMV CP. The translation initiation codon (AUG) was created upstream of the first codon (UGC, which codes for Cys) of the V3 loop so that the full length fusion protein will be read only from this codon. The clone also contained 5'- (37 nucleotides upstream of wild type AlMV CP translation start codon) and 3'-(192 nucleotides following AlMV CP stop codon containing AlMV origin of assembly) noncoding regions of AlMV CP. The segment of pSPCPMNV3 containing the DNA for HIV-1 V3 loop, the AlMV CP, and the 5'- and 3'-noncoding regions of AlMV CP, were excised by *EcoR1* and *Sma1* then ligated into B30Rz, that had been cleaved by *Xho1*, by blunt end ligation. The resulting plasmid was pBRzCPMNV3. This strategy (described for the cloning of V3 loop of HIV-1MN strain) was used to clone the V3 loop, *vpr* and *vpu*, of the HIV-1 NL 4.3 strain. The primers used in PCR reactions to obtain a specific sequences of these genes are listed in Table. 1. The PCR products were cloned into PSPΔAUG linearized by *Xho1* to fuse with AIMV CP and create chimeric protein. The resulting plasmids were named pSPCPNLV3, pSPCPNLVpr, and pSPCPNLVpu. The full length fusion protein carrying V3 loop, *vpr*, or *vpu* was introduced into B30Rz to generate plasmids, pBRzCPNLV3, pBRzCPNLVpr, and pBRzCPNLVpu. These plasmids contain full length TMV molecule and engineered fusion proteins subcloned under the subgenomic promoter of TMV CP.

Table 1

HIV-1 NL 4.3 V3 loop: 5' primer

AGA TCT CGA GAT GAG TTC ATC TGT AGA AAT TAA TTG TACA (SEQ ID NO:5)

HIV-1 NL 4.3 V3 loop: 3' primer

CGG CTC GAG CTA CTA ATG TTA CAA TG (SEQ ID NO:6)

HIV-1 NL 4.3 Vpr: 5' primer

GCA CTC GAG CAG ATG GAA CAA GCC CCA (SEQ ID NO:7)

HIV-1 NL 4.3 Vpr: 3' primer:

GCA CTC GAG GCG GAT CTA ATG GCT CCA TT (SEQ ID NO:8)

HIV-1 NL 4.3 Vpu: 5' primer

GCA CTC GAG GTG ATG CAA CCT ATA ATA GTA (SEQ ID NO:9)

HIV-1 NL 4.3 Vpu: 3' primer:

GCA CTC GAG GCC AGA TCA TCA ATA TCC CA (SEQ ID NO:10)

31 DNV10C primers for rabies N protein (NV10c) and synthetic epitope (31D) presented as DNV10c:

31DNV10C: 5' primer

GCGCTCGAGATGTCCGCCGTCTACACCCGAATTATGATGAACGGAGGACGACTTAAGCG ATACGAGGCAGCTGAAC (SEQ ID NO:11)

31DNV10C: 3' primer;

GCGCTCGAGTCGTCTGCTAGTGCCACGTCGGTAAGGGTAAGTTCAGCTGCCTCGTATCG CTTAAGTCGTCC (SEQ ID NO:12)

31DG24 primers for linear epitope of rabies G protein (rg24) and synthetic peptide (31D) presented as DRG24:

31DG24: 5' primer

GCGCTCGAGATGTCCGCCGTCTACACCCGAATTATGATGAACGGAGGACGACTTAAGCG ACCACCAGACCAGCTTG (SEQ ID NO:13)

31DG24: 3' primer;

GCGCTCGAGTCCTCTTCCACCACAAGGTGCTCATTTTCGTCGTGAAGGT TCACAAGCTGGTCTGGTGGTCGCTTAAGTCGTCC (SEQ ID NO:14)

Construction of fusion proteins consisting of full length AIMV CP and chimeric rabies epitopes: DNV10c and Drg24

DNV10c is a chimera of the linear epitope (NV10c) of rabies nucleocapsid protein and the synthetic peptide 31D. Drg24 is a chimera of the linear epitope (rg24) of rabies glycoprotein and the synthetic peptide 31D. NV10c and rg24 are the B cell determinants. Synthetic peptide 31D is the T cell determinant.

The linear epitopes of the N (NV10C) and G (g24) proteins of rabies virus were engineered as a chimeras with the synthetic peptide 31D and the chimeras fused with AlMV CP. Each chimera (DNV10c and Drg24) was synthesized by PCR using overlapping primers (Table 1) which serve as a template for each other. The primers are made in a way that the first strand and second strand primers has 18 homologous nucleotides that will anneal during PCR reaction. Thus each primer will serve as a template for other one and support the synthesis of new chain. The primers were created to synthesize known

amino acid sequence. The PCR products resulting from these reactions were digested by *Xho*I and cloned into pSPCPΔAUG to fuse with AIMV CP. The resulting plasmids were named as pSPCPDNV10c and pSPCPDrg24. The sequences for full length fusion capsid proteins and for 5'- 3' noncoding regions were cut by *Eco*RI plus *Sma*I and cloned into B30Rz linearized *by Xho*I and subsequent blunt end ligation to create pBRzCPDNV10c, pBRzCPDrg24. All clones, described above (pSPCPMNV3, pSPCPNLV3, pSPCPNLVpr, pSPCPNLVpu, pSPCPDNV10c, and pSPCPDrg24), were subjected to *in vitro* translation and sequencing analysis before they were cloned into final vector (B30Rz).

Construction of chimeric ACP/TMV.

To engineer a chimeric TMV encapsidated with AlMV CP we used the TMV vector (Av/TMV) that had a nontranslatable coat protein. The Av/TMV was created in the laboratory of William Dawson of Florida University. The plasmid was derived from B30Rz used for the cloning described above. The schematic representation of the plasmid is given on Fig. 1A. To engineer the chimeric virus the wild type or recombinant AlMV CP (CP carrying Drg24) was cloned so as to be under the control of TMV CP subgenomic promoter. The AIMV CP was excised from pSP65A4 (Yusibov and Loesch-Fries, Proc. Natl. Acad. Sci. US 92, 8980 (1995)) by *Eco*RI plus *Sma*I and ligated into Av/TMV digested by *Xho*I by blunt ends to create the pAv/ACP. The pAv/ACPDrg24 was created identical to pBRzCPDrg24 using the primers described above and *Xho*I cloning site.

*In vitro* transcription and translation. *In vitro* transcripts of recombinant genes or recombinant TMV were synthesized using Promega T7 or SP6 RNA polymerase and CsCl purified plasmid DNA. The reaction was performed according to manufacturer guidelines. Transcripts were capped using RNA cap structure analog [m7G(5)ppp(5)G, Biolabs]. The transcripts were assayed by *in vitro* translation to determine the messenger activity of each RNA.

*In vitro* translation reactions were performed using a wheat germ cell-free translation system (Promega) and $^{35}$SMet (DuPont). The reactions were carried out as described by the manufacturer and the resulting products were separated by electrophoresis in a 13% SDS-polyacrylamide gel followed by autoradiography.

Preparation, inoculation and immunoassays of protoplasts

Protoplasts were isolated from axenic tobacco plants *(Nicotiana tabacum var. Xanthi-nc)* as described (Yusibov and Loesch-Fries, 1996) and inoculated with 3 μg of recombinant TMV transcripts per 1x10$^5$ protoplasts using a polyethylene glycol procedure (Yusibov and Loesch-Fries, Proc. Natl. Acad. Sci. U.S. 1995). After inoculation the protoplasts were incubated on ice (15 minute) pelleted and washed twice with 10% mannitol. After final speen the protoplasts were resuspended in 1xAIOK medium (0.2 mM $KH_2PO_4$, 1 mM KNQ , 1mM $MgSO_4$, 1 μM KI, 0.1 μM CuSQ , 10 mM $CaCl_2 .2H_2O$, pH 6.5) and incubated at low light conditions at 25-27° C. The protoplasts were collected 24 hr after inoculation and assayed by

immunofluorescence using monoclonal antibodies to AlMV CP (Loesch-Fries & T. Hall, J. Gen. Virol., 47, 323 (1980))to determine the accumulation of recombinant protein (immunofluorescent microscopy or Western analysis).

Immunoprecipitation of particles.

The particles extracted from plant tissue which were coinfected with transcripts of recombinant virus were immunoprecipitated using monoclonal antibodies to the linear epitope of rabies G protein (rg24). Antibodies (Dietzschold et al., Virology 64, 3804 (1990)) were mixed with recombinant virus in a ratio 1: 500 (w:w) and incubated at 4° C with agitation for two hours. Within 2 hr the suspension of (50 μl) formalin-fixed staph A cells were add to the incubation mix and continued incubate at the same conditions for one more hour. After incubation was complete the cells were pelleted and washed three time with the original buffer in which the virus particles were stored (sodium phosphate buffer, pH 7.2). The final pellet was resuspended in a 50 μl of protein loading buffer and used for the Western analysis.

Western analysis. Protein preparations from virus infected tissue, purified virus samples or from immunoprecipitation were separated on SDS-PAGE electrophoresis and electroblotted on to nylon membrane using Towbins transfer buffer (0.025 M Tris, 0.192 M glycine, 20% methanol, pH 8.3) overnight at 33 mA. After blocking with milk (Kierkegarden) proteins were reacted with appropriate antibodies Westatin stain kit manufacturer (Sigma)).

Plant infection and virus isolation

Primary infection of tobacco leaves was initiated with *in vitro* transcription products of recombinant TMV strains, described above. Transcription products of recombinant virus were diluted 1:1 (final concentration: 15 mM) in 30 mM sodium phosphate pH 7.2 and applied to expending tobacco leaves (growing, 3-4 week old leaves). Inoculation was effected by gentle rubbing in the presence of carborundum (320 grit; Fisher, Pittsburgh, PA) to spread the inoculum and abrade the leaf surface. Inoculum was applied after the abrasive. Inoculated *N. bentamiana* plants were isolated in a greenhouse and maintained with normal watering and fertilization. To isolate the virus particles carrying recombinant protein from locally and systemically infected leaves of tobacco they were harvested 12 days post-inoculation. The leave tissue was frozen in liquid nitrogen and ground in prechilled mortar. Ground tissue was transferred into sterile tubes containing buffer (I ml/1 g of tissue; 0.25 M sodium phosphate, pH 7.2) and resuspended by vortexing followed by centrifugation at 10,000 rpm for 15 minutes. All manipulations with samples were performed at 0 to 4° C. Upon centrifugation the supernatant was transferred into new tubes and virus particles were selectively precipitated in a buffer containing 4% polyethylene glycol (MW 15,000 - 20,000) and 50 mM NaCl for 2 hours. Polyethylene glycol is a component that precipitates virus particles. Then virus particles were pelleted at 10,000 rpm for 20 minutes. The pellet was resuspended in a 25 mM sodium phosphate buffer pH 7.2 and centrifuged once again

under a similar conditions to separate possible plant debris and insoluble plant components. The supernatant (which contains virus) from this step was used for future experiments.

## Example 2

**Synthesis of fusion capsid proteins**

*In vitro* translation of fusion capsid proteins

Before cloning into the final vector (30BRZ) the recombinant genes were tested for the presence of a complete open reading frame of fusion capsid proteins by sequencing and/or by *in vitro* translation. Sequence analysis was performed using CsCl purified plasmid DNA containing original PCR fragments (pSPCPMNV3, pSPCPNLV3, pSPCPNLVpr, pSPCPNLVpu, pSPCPD10c, and pSPCPDrg24) and SP6 primer. CsCI purified recombinant plasmid (pSPCPMNV3, pSPCPNLV3, pSPCPNLVpr, pSPCPNLVpu, pSPCPD10c, and pSPCPDrg24). DNA containing engineered genes was digested by *Sma*I and used for the *in vitro* transcription. The capped transcripts of recombinant genes were synthesized using SP6 polymerase and translated in a wheat germ cell free translation system as described above. All tested transcripts had a messenger activity and directed the incorporation of $^{35}$SMet into polypeptides of expected size.

Translation of fusion capsid proteins in infected tobacco protoplasts

To assess the expression of fusion capsid proteins from TMV vector, the full length capped transcripts of recombinant virus were made and used for infection of tobacco protoplasts. 24 hr after inoculation with 3 $\mu g$ of transcripts per $1x10^5$ , the protoplasts were collected and used for immunoassay and for the Western analysis. Immunofluorescent assay of fixed protoplasts where we used antibodies (Loesch-Fries and T. Hall, J. Gen. Virol., 47, 323 (1980) against AlMV CP for detection showed a significant amount of protein accumulation in an individual infected cell (Data not shown). To assess the size of expressed proteins and their reaction with specific antibodies the proteins were separated on SDS polyacrylamide gel, transferred to a nylon membrane, and reacted with the monoclonal antibodies to each peptide (results not shown) or to the AlMV CP (Fig. 2). All fusion capsid proteins migrated in a range of expected size (28-35 kDa) and reacted with monoclonal antibodies to the AlMV CP or to specific peptides. The difference in the size of fusion proteins is dictated by the difference in the size of each fused with AlMV CP peptide.

Expression of fusion capsid protein in infected plants

To assess the expression of recombinant protein in locally and systemically infected plant tissues the expending leaves of tobacco were inoculated with transcripts of recombinant TMV. Twelve days after inoculation, the virus was purified from locally and systemically infected leaves

separately. In local infections, infection occurred in originally inoculated leaves. In systemic infection, the spread of virus was throughout the plant into new growing noninoculated leaves. Prior to purification 30-50 mg of infected tissue was used to determine if, together with TMV particles, the recombinant AlMV particles were assembled. The tissue was homogenized and the sap from it was applied on a carbon coated grid. The electron micrograph shows that spherical particles (presenting recombinant AlMV) were assembled upon infection with transcripts from all constructs (pBRzCPMNV3, pBRzCPNLV3, pBRzCPNLVpr, pBRzCPNLVpu, pBRzCPDNV10c, and pBRzCPDrg24). Fig.3 presents the results of negative staining of particles (wild type B30Rz and recombinant BRzCPDrg24) using 2% urea acetate.

Western blot analysis of purified virus samples demonstrated the presence of fusion capsid proteins in samples from both locally and systemically infected leaves (Fig. 4). This indicated that the recombinant virus was viable and it retained the fusion capsid protein during systemic movement through the plant.

Infection of tobacco plants with pAv/ACP and pAv/ACPDrg24.

The *in vitro* transcripts of pAv/ACP or pAv/ACPDrg24 were used for inoculation of tobacco plants. Within eight days the tissue samples were collected to assess the systemic spread of virus in noninoculated leaves. Western analysis (data not shown) detected the wt (ACP) or recombinant (ACPDrg24) protein in a systemic noninoculated leaves of tobacco indicating that

AlMV CP supported the systemic spread of CP defective TMV. The plants inoculated with transcripts of the vector itself (pAv/TMV) did not show any systemic symptoms on tobacco plants even 20 days after inoculation.

Assembly of recombinant AlMV particles presenting epitopes from different pathogens

Samples of infected leaf tissues from pBRzCPMNV3, pBRzCPNLV3, pBRzCPDNV10c, and pBRzCPDrg24-infected plants were taken, combined and used for the infection of tobacco plants. The virus particles were purified 12 days after inoculation and assessed for co-assembly of recombinant AlMV CPs from different constructs. The virus particles were immunoprecipitated using monoclonal antibodies to the linear epitope of rabies G protein (rg24) and formalin fixed Staph A cells. Immunoprecipitation products were separated by SDS polyacrylamide gel electrophoresis (above) and used for the Western blot analysis. The separated proteins were reacted to the monoclonal antibodies for the AlMV CP, antibodies against the linear epitopes of rabies N and G proteins, and antibodies against V3 loop of HIV1 MN strain (National Institute of Allergy and Infectious Diseases. AIDS Research and Reference Reagent Program. #1728 Antibody to HIV-1 V3). All antibodies reacted with the immunoprecipitation product after gel separation (Fig. 5) indicating that upon co-infection AlMV CP molecules will assemble into multivalent particles presenting antigenic epitopes from different pathogens. The larger molecular weight bands seen in Fig. 5

represent protein dimers similar to those of control virus (AlMV CP).

**Example 3**

**Immunization of mice with AlMV/TMV construct expressing the Drg24 peptide epitope of rabies glycoprotein**

Eight-week old female Swiss-Webster, outbred mice were immunized with 10 μg per dose of recombinant TMV virus engineered to express the rg24 epitope of rabies glycoprotein (Drg24-A/TMV). Three immunizations of 0.1 ml were administered intra-peritoneally at intervals of 2 weeks each with and without complete Freund's adjuvant (CFA) at a 1:1, vol:vol ratio. An equal quantity of a mixture of wild type AlMV plus TMV was used with and without CFA as controls. Ten-to-fourteen days after each immunization, serum samples were obtained from individual mice and rabies virus-specific antibody titers assessed. Antigen-specific antibody analysis of serum was performed using a solid phase enzyme-linked immunoabsorbant assay (ELISA). ELISA plates (Nunc Polysorp, Denmark) were coated with 100 μl per well of inactivated ERA-strain rabies virus (5 μg/ml in Phosphate-buffered saline) overnight at room temperature (RT; about 25 °C). Coated plates were washed 3x with PBS-Tween (0.05%) and then blocked with 5% dried milk in PBS at RT for at least 1 hour. A series of dilutions of sera were added to the plates (30 μl/well) for 2 to 4 hours at RT. The plates were then washed 3x with PBS-Tween and peroxidase-conjugated secondary antibodies (goat

anti-mouse IgG, either whole molecule or gamma chain specific) were added (100 μl per well) at a final dilution of 1:2000 in PBS, for 1 hour at RT. Plates were then washed 5x with PBS-Tween and TMB substrate added (100 μl/well) in phosphate-citrate buffer containing urea, for 30 min at RT in the dark. The reaction was stopped with 2M $H_2SO_4$ (50 μl per well) and the color change resulting from bound specific antibody measured at 450 nM in an ELISA plate-reader (Bio-Tek, Winooski VT). The results, expressed in O.D. units, are shown (Fig. 6A). Eighty percent of mice immunized with particles carrying Drg24 had rabies-specific antibodies (Fig. 7). Specific neutralization of rabies virus was assessed using a modified rapid fluorescent focus forming assay (Fig. 6B). Serum was inactivated by treatment for 30 minutes at 56°C and diluted in MEM medium supplemented with 10% fetal bovine serum (FBS) to a starting dilution of 1/5. The 1/5 serum dilution was further diluted serially 1/2 (1 volume plus 1 volume diluent) in 96 well plates (Nunc) such that each well contained 50 μl of the titrated serum. Thirty μl of a preparation of rabies CVS-11 virus was added to each well. The rabies virus solution was prepared such that 30 μl diluted with 50 μl of medium and 30 μl of BHK indicator cells (1.5 x $10^6$/ml) contained sufficient virus to cause infection of 80 to 90% of the cells in monolayer cultures after 20 hours. The 96-well plates containing the serum dilutions and rabies virus were incubated for 1 hour at 37°C prior to being carefully mixed with 30 μl of BHK indicator cells (1.5 x 106/ml). Ten μl of each of these mixtures was transferred to the wells of

Terasaki plates (Nunc). The Terasaki plates were incubated for 20 hours at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. The plates were then washed 3x with PBS and the cells fixed by the addition of ice-cold acetone (90%) for 20 minutes. The plates were then air dried and 5 $\mu$l of a 1/40 dilution of fluorescein-conjugated rabies virus-specific antibody (Centocor) added to each well for 40 min. at 37°C. The plates were then washed 3x with water and the percentage of infected BHK cells evaluated using a fluorescent microscope (Leitz). Fig. 6B shows the presence of neutralizing antibodies in sera.

**Example 4**

**AlMV constructs not involving TMV nucleic acid**

AlMV constructs free of TMV RNA analogous to those described above are constructed in a matter analogous to the TMV recombinant constructs. AlMV nucleic acid is substituted for the TMV RNA. The structure of the AlMV genome is published and the required functions coded for by the AlMV genome have been mapped. (Bol et al., Virology 46, 73 (1971); Bol et al., Virology 58, 101 (1974)).

**Example 5**

**Immunization of mice with AIMV/TMV construct containing recombinant AIMV CP carrying chimeric rabies peptide Drg24 and challenge of immunized mice with CVS-24 strain rabies virus.**

Eight week old female Swiss-Webster, outbred mice were immunized with 50 mg per dose of recombinant TMV virus engineered to express the recombinant AlMV CP (CPDrg24) carrying chimeric epitome (DRg24) of rabies virus. Three immunizations of 0.1 ml were administered intra-peritoneally at intervals of 2 weeks. No adjuvant was used in this experiment. An equal quantity of a mixture of wild type AMV plus TMV was used as a control. Ten-14 days after each immunization serum samples were obtained from individual mice and rabies virus-specific antibody titers were assessed. Antigen-specific antibody analysis of serum was preformed using a solid phase enzyme-linked immunoabsorbant assay (ELISA) as described in Example 3.

Specific neutralization of rabies virus was assessed using a modified rapid fluorescent focus forming assay. The assay was performed as described in Example 3 using CVS-11 strain rabies virus (Table 2). Fourteen days after the third immunization the groups of mice (10 mice in a group) were challenge inoculated with a deadly dose equal to approximately ten times the IMLD50 ("intramuscular lethal dose 50") of CVS-24 strain rabies virus. All control mice (nonimmunized and mice immunized with vector only) died by day 6 to 7 while 40% of CPDrg24 immunized mice were protected. 60% of CPDrg24 immunized mice died by day 15-16. However, all immunized mice survived longer than either the control or nonimmunized mice, indicating some level of protective immunity for all immunized

mice. The results of the challenge experiment are presented in Table 2.

**Example 6**

**Immunization of mice with an AlMV/TMV construct containing recombinant AlMV CP carrying the V3 loop of HIV-1 MN strain.**

Eight week old female Swiss-Webster, outbred mice were immunized with 10 mg per dose of recombinant TMV virus engineered to express the recombinant AlMV CP (CPMNV3) carrying V3 loop of HIV-1 MN strain. Seven immunizations of 0.1 ml were administered intra-peritoneally at intervals of 2 weeks. Three immunizations of 0.1 ml were administered intra-peritoneally at intervals of 2 weeks each with and without complete Freund's adjuvant (CFA) at 1:1, vol:vol ratio. An equal quantity of a mixture of wild type AMV plus TMV (30B/AlMV in Fig. 8) was used with and without CFA as a control. Ten-14 days after each immunization serum samples were obtained from individual mice and rabies virus-specific antibody titers were assessed. Antigen-specific antibody analysis of serum was performed using a solid phase enzyme-linked immunoabsorbant assay (ELISA) as described in Example 3. Sera from mice immunized with CPMNV3 were assessed for the presence of antibodies specific for the synthetic peptide derived from the V3 loop of HIV-1. Low levels of serum antibodies specific for HIV were detectable after the third inoculation. Fourteen days after the last immunization with

CPMNV3 serum antibodies specific for the V3 loop of the HIV-1 MN isolate were detected in both ELISA and neutralization assays as shown in Figure 8A, 8B, and 8C. While preimmune sera had low neutralizing activity (about 25%) at dilutions 1:128 and 1:256, sera from the experimental mice inoculated with CPMNV3 demonstrated, on average, approximately 80% and 76% neutralizing activity at the same dilutions respectively (Fig. 8C).

The HTLV-I/MT2 cell lines (obtained from the NIH AIDS Research and References Reagent Program) were used as target cells in the HIV-1 neutralization assay. These cells were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin-streptomycin and pyruvate. The cell-free HIV-1/MN isolate was propagated in HTLV-1/MT2 cells as described. Cell-free virus (100 TCID50) was preincubated with different dilutions of heat-inactivated preimmune or immune sera for 1 hr at 37° C. Following incubation, the serum-treated virus was used to infect HTLV-I/MT2 cells. Syncytia formation was evaluated 5 days after inoculation of HTLV-I/MT2 cells by phase contrast microscopy.

**Example 7**

**Oral immunization of mice with AMV/TMV construct containing recombinant AIMVCP carrying chimeric rabies peptide Drg24.**

Eight week old female Swiss-Webster, outbred mice were immunized via gastric intubation with 250 mg per dose of recombinant TMV virus engineered to express the AlMV CP (CPDrg24) carrying chimeric epitope (Drg24) of rabies virus. Five immunizations of 0.1 ml were administered orally at intervals of 2 weeks. No adjuvant was used in this experiment. An equal quantity of a mixture of wild type AMV plus TMV (A/TMV in **Fig. 9A** and **9B**) was used as a control. Ten-14 days after each immunization serum samples were obtained from individual mice and rabies virus-specific antibody titers assessed. Antigen-specific antibody analysis of serum was performed using a solid phase enzyme-linked immunoabsorbant assay (ELISA) as described in Example 3. The increasing levels of rabies virus-specific IgG and IgA were detected in the sera of immunized mice (**Figs. 9A** and **9B**).

Table 2

Neutralization titers of sera from the mice immunized with CPDrG24 and challenge infection of these mice with CVS-24 strain rabies virus.

| Groups of mice | Neutral-ization titers (mean titer) | Challenge with CVS-24 Strain Rabies Virus | | | |
|---|---|---|---|---|---|
| | | Days after challenge (dead/total) | | | SURVIVALS |
| | | 6-7 | 10-11 | 15-16 | |
| Mice immunized with CPDrg24 | 165 | 0/10 | 5/10 | 6/10 | 4/10 |
| Mice immunized with A/TMV | 0 | 10/10 | 0 | 0 | 0 |
| Non immune mice | 0 | 10/10 | 0 | 0 | 0 |

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Koprowski, Hilary
Yusibov, Vidadi
Hooper, Douglas C.
Modelska, Anna

(ii) TITLE OF INVENTION: Polypeptides Fused with Plant Virus Coat Proteins

(iii) NUMBER OF SEQUENCES: 16

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE:
(B) COMPUTER:
(C) OPERATING SYSTEM:
(D) SOFTWARE:

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME:
(B) REGISTRATION NUMBER:
(C) REFERENCE/DOCKET NUMBER: TJU-PT004PC

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(A) TELEPHONE: (215) 568-6400
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(2) INFORMATION FOR SEQ ID NO:1

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Thr Arg Pro Asp Tyr Asp Lys Arg Lys Arg Ile His Ile Gly Pro
1 5 10 15

Gly Arg Ala Phe Tyr Thr Thr Lys Asp Ile Ile Gly Thr Ile Arg Gln
20 25 30

Ala His Cys
35

(2) INFORMATION FOR SEQ ID NO:2

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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1 5 10 15

Gly Pro Gly Arg Ala Phe Val Thr Ile Gly Lys Ile Gly Asp Met Arg
20 25 30

Gln Ala His Cys
35

(2) INFORMATION FOR SEQ ID NO:3

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ser Ala Val Tyr Thr Arg Ile Met Met Asp Gly Gly Arg Leu Lys
1 5 10 15

Arg Tyr Glu Ala Ala Glu Leu Thr Leu Thr Asp Val Ala Leu Ala Asp
20 25 30

Asp Ser

(2) INFORMATION FOR SEQ ID NO:4

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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1 5 10 15

Arg Pro Pro Asp Gln Leu Val Ala Leu His Asp Gly Ile Glu Lys Leu
20 25 30

Val Val Glu Glu Asp Ser
35

(2) INFORMATION FOR SEQ ID NO:5

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGA TCT CGA GAT GAG TTC ATC TGT AGA AAT TAA TTG TAC A 40

(2) INFORMATION FOR SEQ ID NO:6

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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(2) INFORMATION FOR SEQ ID NO:7

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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(2) INFORMATION FOR SEQ ID NO:8

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCA CTC GAG GCC AGA TCA TCA ATA TCC CA 29

(2) INFORMATION FOR SEQ ID NO:11

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 76 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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(2) INFORMATION FOR SEQ ID NO:12

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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TTAAGTCGTC C 71

(2) INFORMATION FOR SEQ ID NO:13

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 76 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCGCTCGAGA TGTCCGCCGT CTACACCCGA ATTATGATGA ACGGAGGACG ACTTAAGCGA 60

CCACCAGACC AGCTTG 76

(2) INFORMATION FOR SEQ ID NO:14

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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TCTGGTGGTC GCTTAAGTCG TCC 83

(2) INFORMATION FOR SEQ ID NO:15

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: amino acid
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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            20                  25                  30

His Phe Pro Arg Ile Trp Leu His Asp Leu Gly Gln His Ile Tyr Glu
        35                  40                  45

Thr Tyr Gly Asp Thr Trp Ala Gly Val Glu Ala Ile Ile Arg Ile Leu
    50                  55                  60

Gln Gln Leu Leu Phe Ile His Phe Arg Ile Gly Cys Arg His Ser Arg
65                  70                  75                  80

Ile Gly Val Thr Arg Gln Arg Arg Ala Arg Asp Gly Ala Ser Arg Ser
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(2) INFORMATION FOR SEQ ID NO:16

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

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1               5                  10                  15

Ile Ala Ile Val Val Trp Ser Ile Val Ile Ile Glu Tyr Arg Lys Ile
            20                  25                  30

Leu Arg Gln Arg Lys Ile Asp Arg Leu Ile Asp Arg Leu Ile Glu Arg
        35                  40                  45

Ala Glu Asp Ser Gly Asp Glu Ser Glu Gly Glu Val Ser Ala Leu Val
    50                  55                  60

Glu Met Gly Val Glu Met Gly His His Ala Pro Trp Asp Ile Asp Asp
65                  70                  75                  80

Leu
81
```

* * *

**CLAIMS**

1. A process of administering a polypeptide to an animal, the process comprising the steps of:

(1) infecting a plant cell with recombinant plant virus nucleic acid that will be processed in a plant cell to produce a fusion capsid protein, said fusion protein comprising a plant virus capsid protein and a polypeptide that is not a plant virus capsid protein, said plant virus capsid protein being an AlMV capsid protein or ilarvirus capsid protein, thereby creating a infected cell;

(2) cultivating the infected cell, or a derivative cell derived from said infected cell, under conditions where said infected cell or derivative cell makes the fusion capsid protein; and

(3) administering the fusion capsid protein or a portion thereof to an animal.

2. The process of Claim 1 wherein the recombinant plant virus nucleic acid comprises TMV nucleic acid.

3. The process of Claim 1 wherein the result of the process is protective immunity.

4. The process of Claim 1 wherein in step (3) the fusion capsid protein is part of a plant or plant material.

5. A process of Claim 1 wherein in step (3) the fusion capsid protein is extracted in purified form from the plant

and administered as a protein substantially free of other compounds of the plant.

6. A process of Claim 1 wherein the polypeptide is a rhabdovirus protein.

7. A process of Claim 1 wherein the polypeptide is a human immunodeficiency virus protein.

8. A process of Claim 12 wherein the animal is a mammal.

9. A process of claim 1, the process comprising the steps of:

(1) infecting a plant cell with a plurality of recombinant plant virus nucleic acids that will be processed in a plant cell to produce a plurality of fusion capsid proteins, each comprising a different polypeptide that is not a plant virus capsid protein, and each further comprising an AlMV capsid protein or ilarvirus capsid protein, thereby creating a infected cell;

(2) cultivating the infected cell, or a derivative cell derived from said infected cell, under conditions where said infected cell or derivative cell makes the fusion capsid proteins; and

(3) administering the fusion capsid proteins or a portion thereof to an animal.

10. A process for producing a polypeptide, the process comprising the steps of:

(1) infecting a plant cell with recombinant plant virus nucleic acid that will be processed by a plant cell to produce a fusion capsid protein comprising an AlMV or ilarvirus capsid protein and the polypeptide, said polypeptide not a plant virus capsid protein, thereby creating a infected cell;

(2) cultivating the infected cell, or a derivative cell derived from said infected cell, under conditions where said infected cell or derivative cell makes the fusion capsid protein.

11. A recombinant virus with a tripartite fusion capsid protein, the fusion capsid protein comprising a capsid protein of a virus that is not AlMV or an ilarvirus, a capsid protein or AlMV or an ilarvirus, and a polypeptide that is not a plant virus polypeptide.

12. The process of Claim 1 wherein the result of the process is an immune response against the polypeptide that is part of the fusion capsid protein but is not a plant virus protein.

13. The process of Claim 12 wherein the immune response is the production of antibodies against the polypeptide.

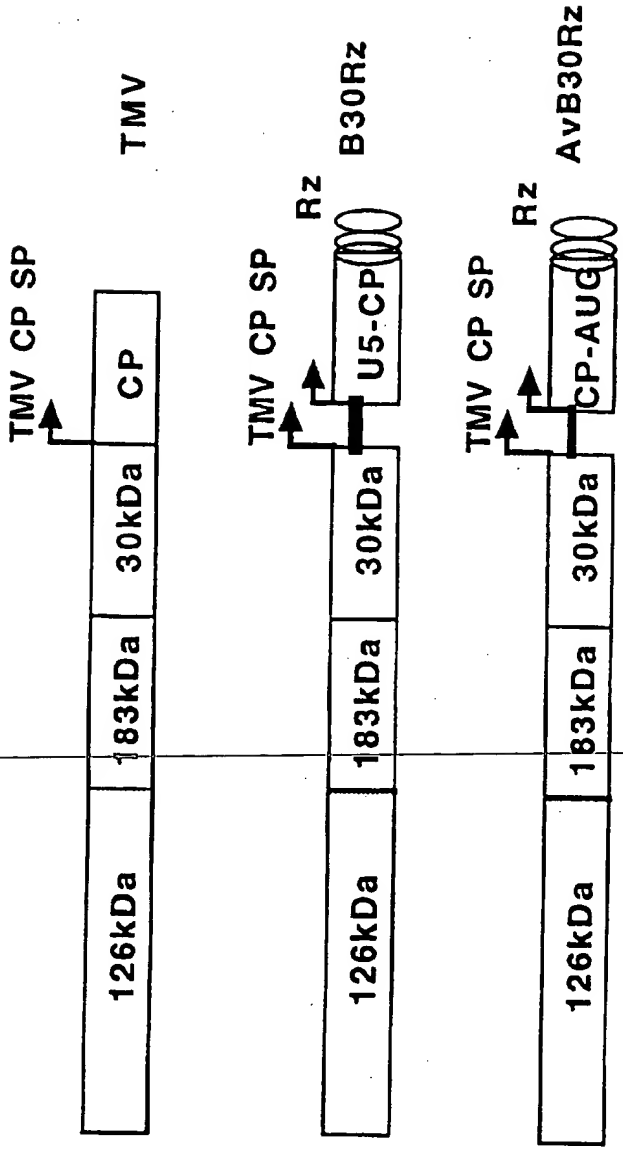



Fig. 1A

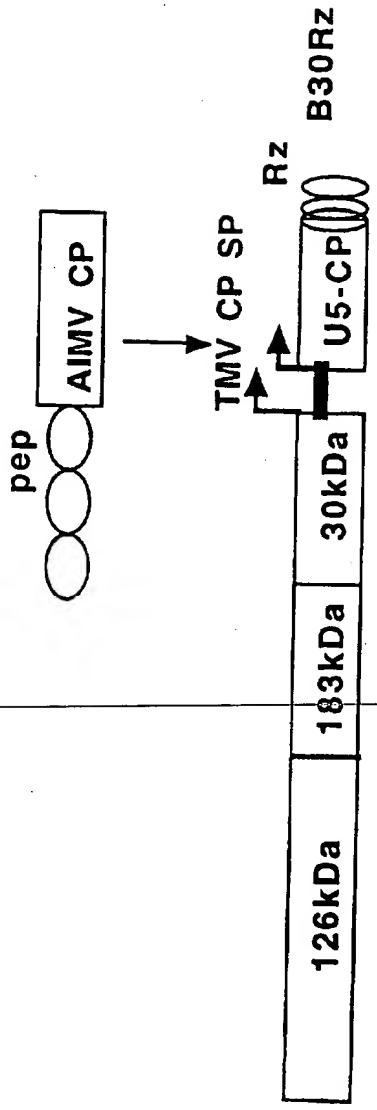



Fig. 1B

Fig. 2

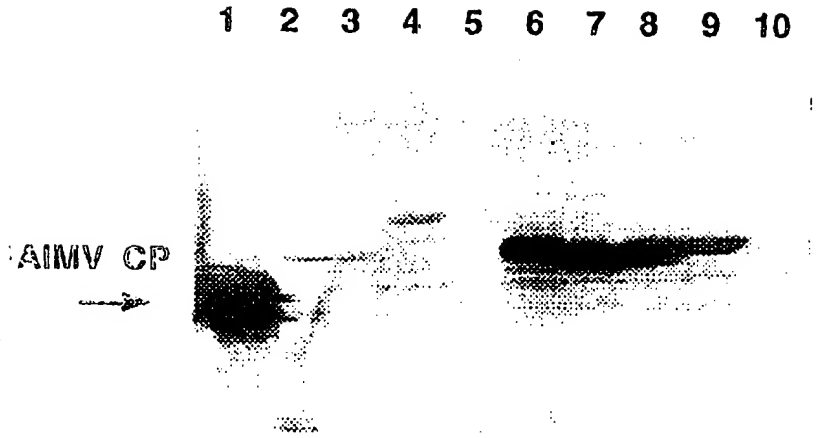


Fig. 3A

Fig. 3B

Fig. 4

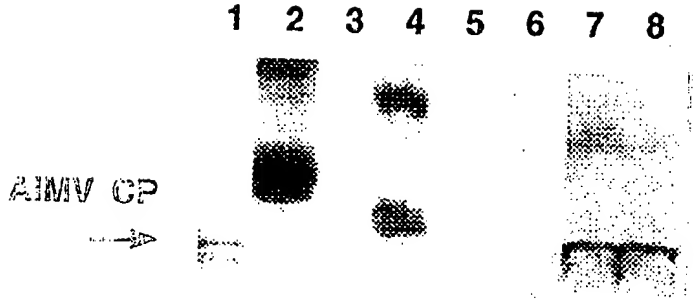
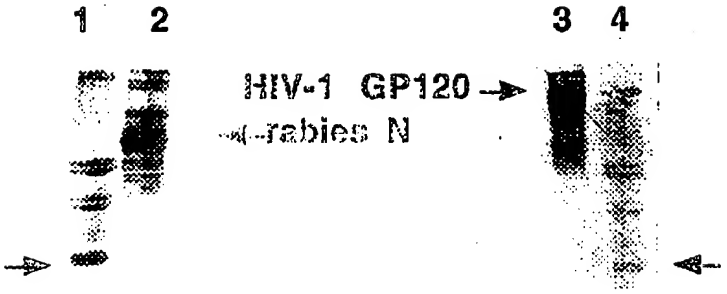


Fig. 5



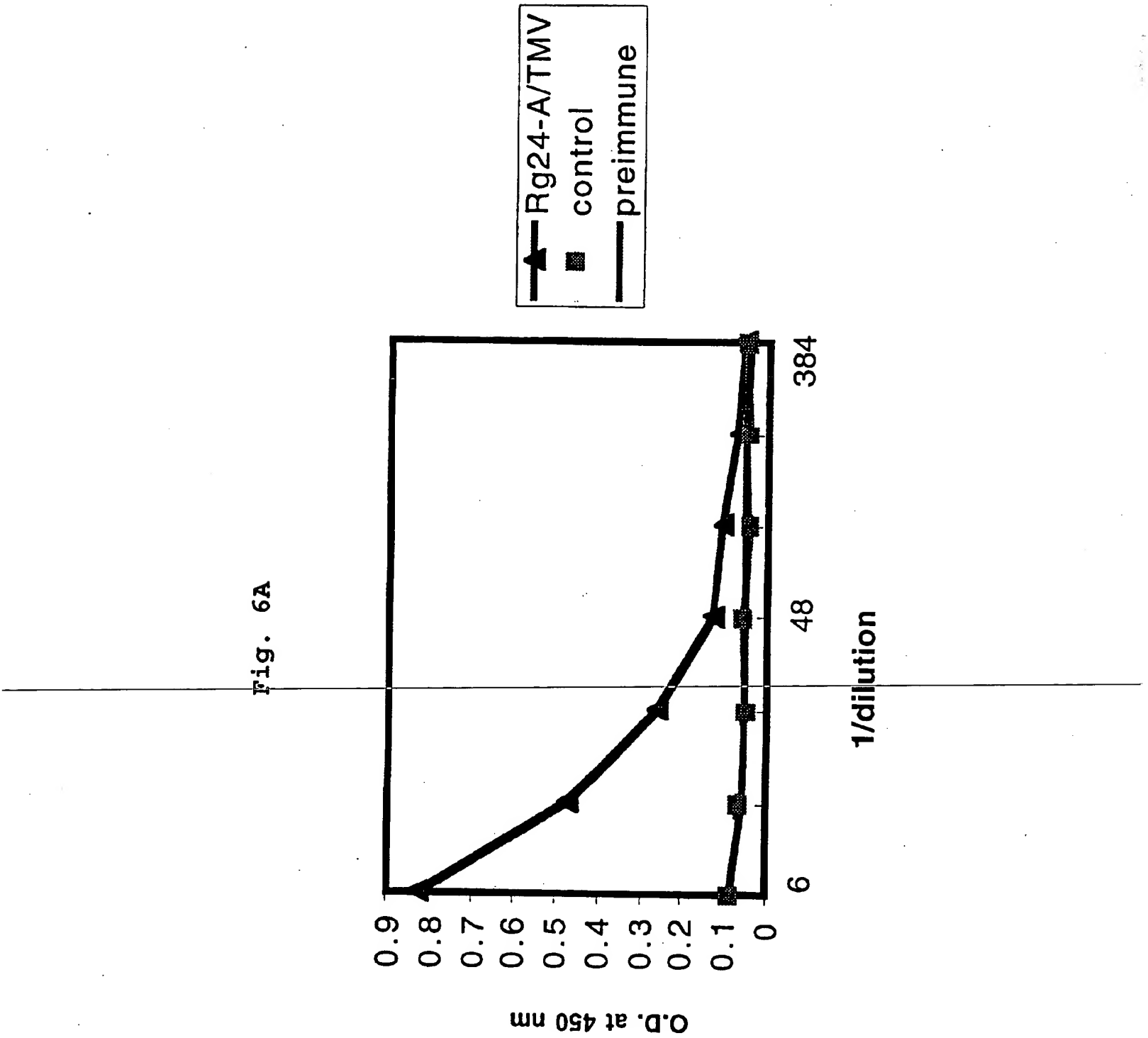



Fig. 6A

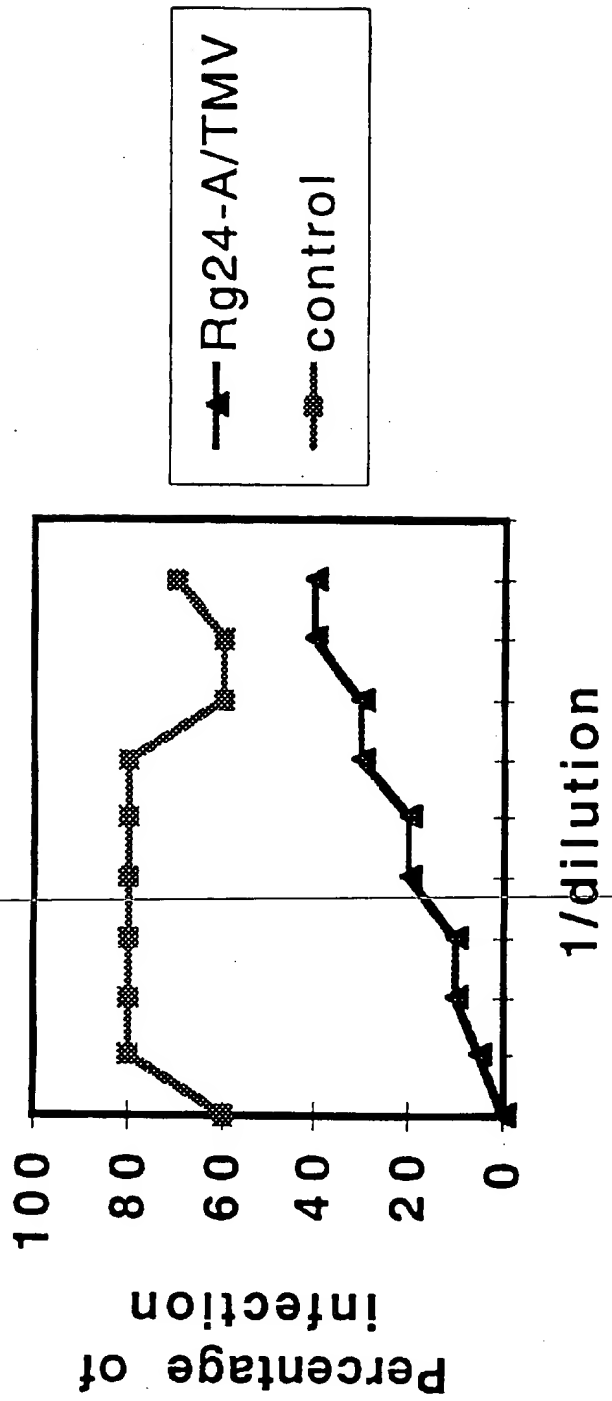



Fig. 6B

Fig. 7

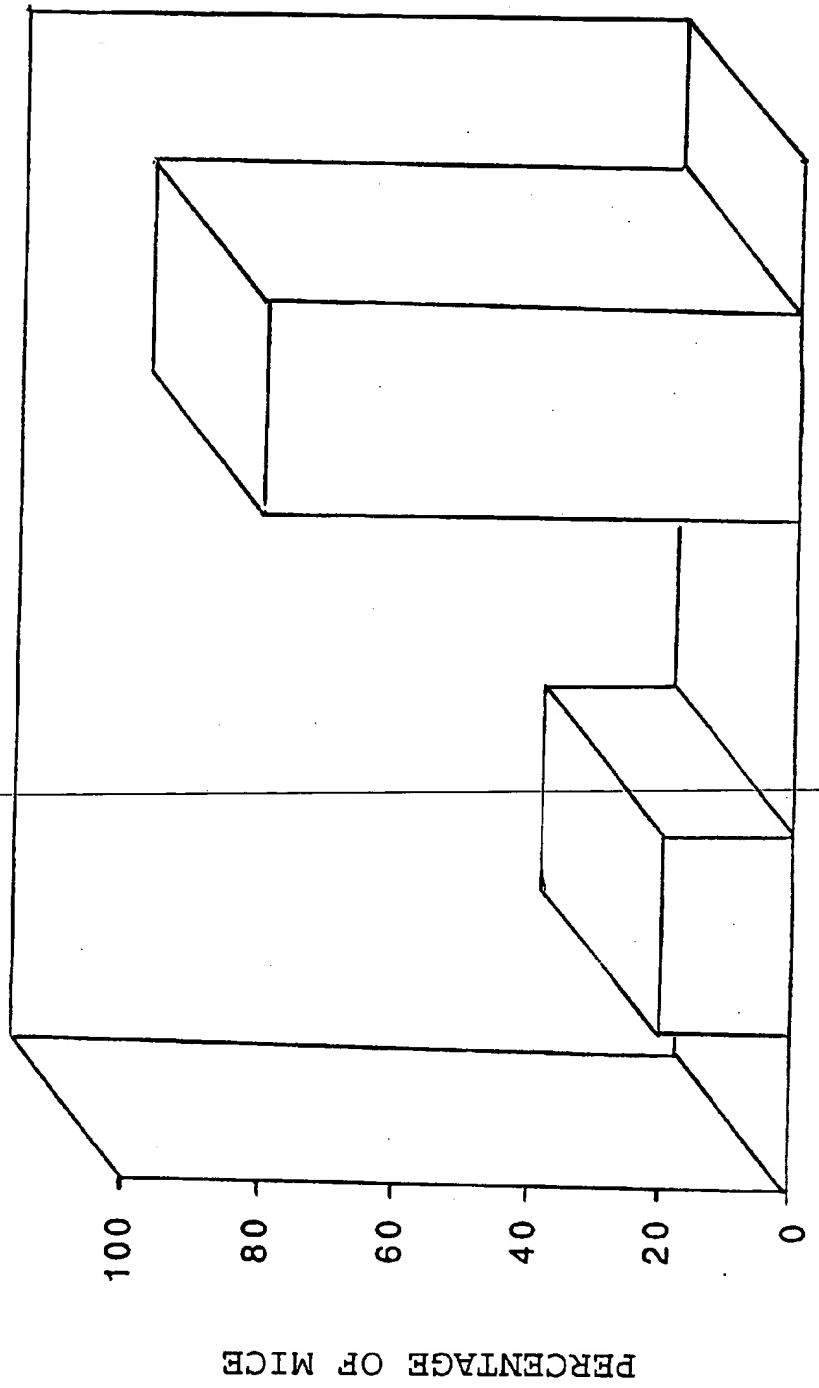


Fig. 8A

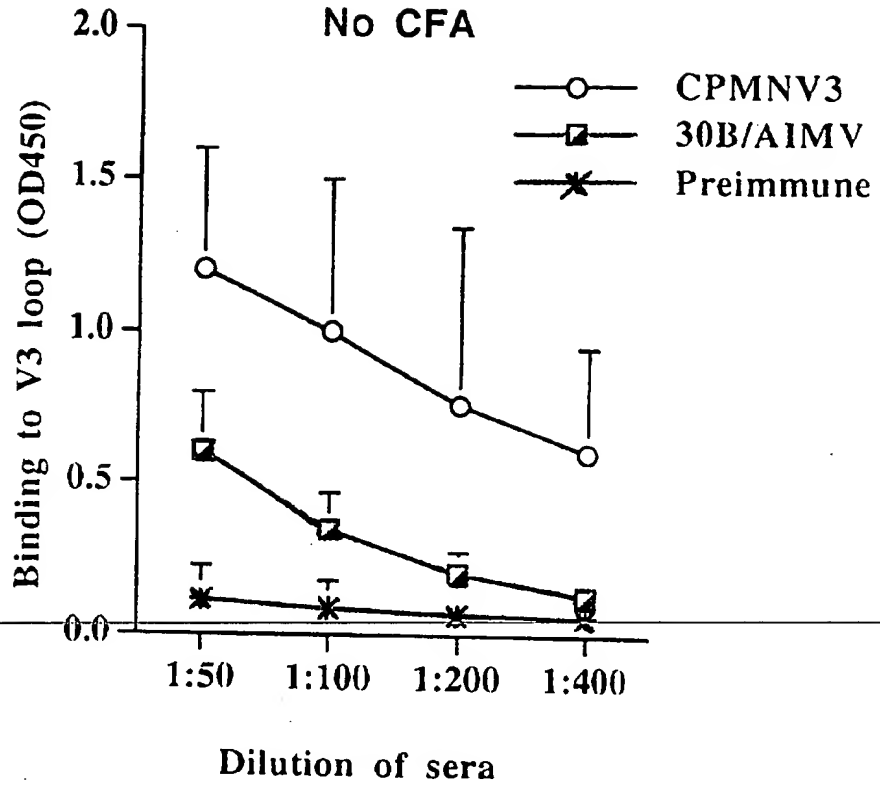


Fig. 8B

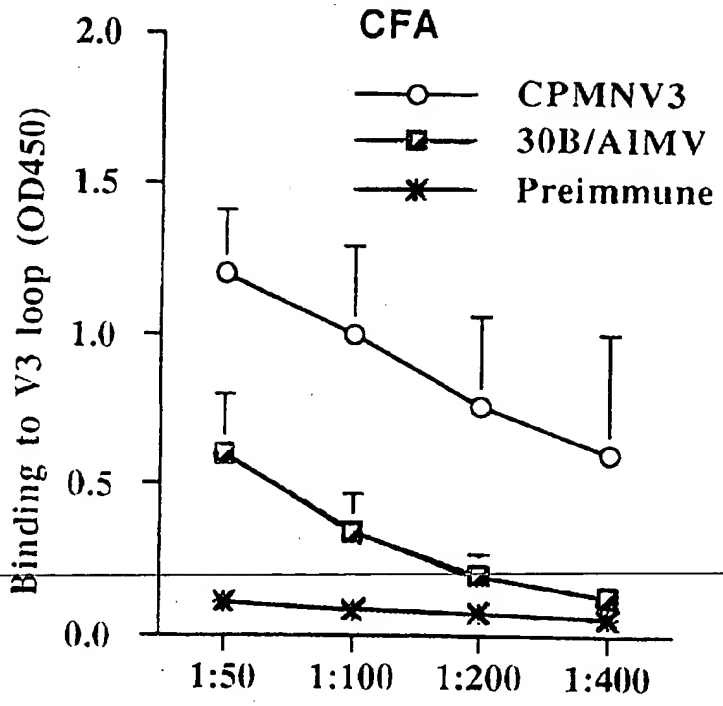


Fig. 8C

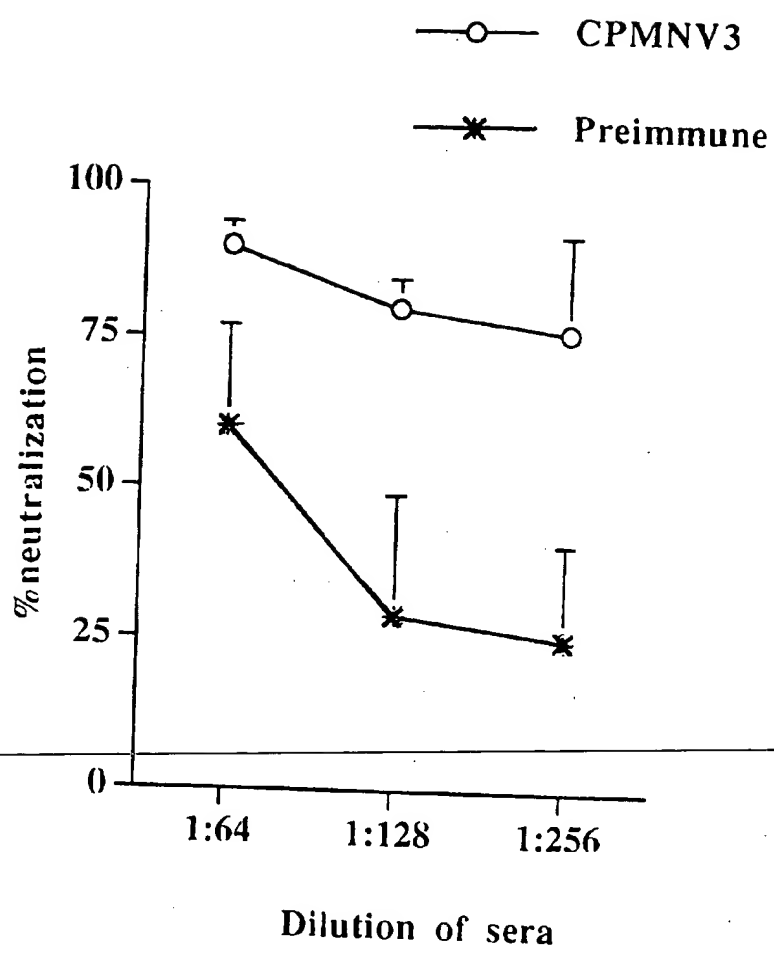


Fig. 9A

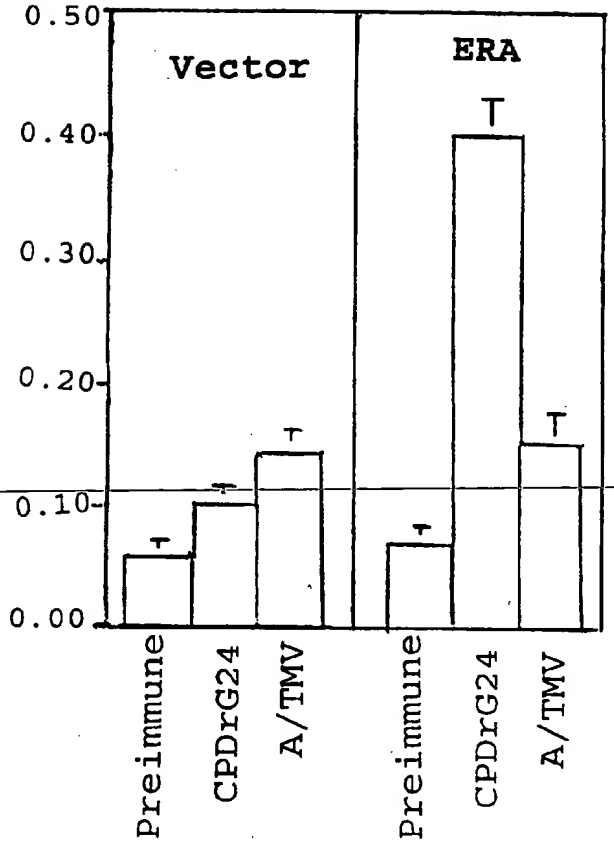
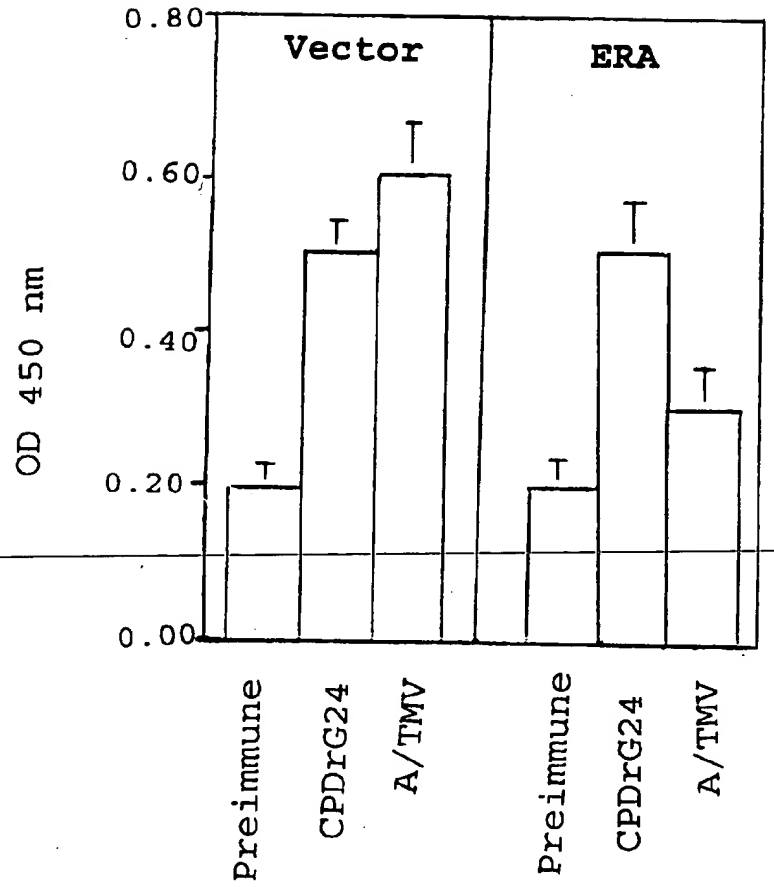


Fig. 9B



# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15200

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A01H 4/00; A61K 39/00; C07K 14/00
US CL :800/200, 205; 424/224.1, 207.1; 435/414, 419, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/200, 205; 424/224.1, 207.1; 435/414, 419, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 92/18618 A1 (LOMONOSSOFF et al.) 29 October 1992, see entire article. | 1-13 |
| Y | BAER et al. Specific RNA binding by amino-terminal peptides of alfalfa mosaic virus coat protein. The EMBO Journal. 1994, Vol. 13, No. 3, pages 727-735, see Abstract. | 1-13 |
| | | |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
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| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>04 NOVEMBER 1997 | Date of mailing of the international search report<br>07 JAN 1998 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>PHUONG BUI<br>Telephone No. (703) 308-0196 |

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